Substrate Specificity of Human Class I Alcohol Dehydrogenase Homo- and Heterodimers Containing the β_2 (Oriental) Subunit†

Wing Ping Fong and Wing Ming Keung*
Department of Biochemistry, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong
Received February 26, 1987; Revised Manuscript Received April 14, 1987

ABSTRACT: In order to gain a better understanding of the metabolism of ethanol in Orientals, the kinetic properties of human alcohol dehydrogenase (ADH) isozymes containing the β_2 (Oriental) subunit, i.e., $\alpha\beta_2$, $\beta_2\gamma_1$, $\beta_2\beta_2$, $\beta_2\gamma_2$, as well as $\gamma_1\gamma_1$, were examined by using primary and secondary alcohol substrates of various chain lengths and compared with those of the corresponding β_1 (Caucasian) subunit containing isozymes already on record [Wagner, F. W., Burger, A. R., & Vallee, B. L. (1983) *Biochemistry 22*, 1857–1863]. With primary alcohols, these isozymes follow typical Michaelis–Menten kinetics with a preference for long-chain alcohols, as indicated by K_m and k_{cat}/K_m values. The k_{cat} values obtained with primary alcohols, except methanol, do not vary greatly, i.e., less than 3-fold, whereas the corresponding K_m values span a 3600-fold range, i.e., from 26 μM to 94 mM, indicating that the specificity of these isozymes manifests principally in substrate binding. As a consequence, ethanol—which might be thought to be the principal in vivo substrate for ADH—is oxidized rather poorly, i.e., from 50- to 90-fold less effectively than octanol. Secondary alcohol oxidation by the homodimers $\beta_2\beta_2$ and $\gamma_1\gamma_1$ also follows normal Michaelis–Menten kinetics. Again, values of K_m and k_{cat}/K_m reveal that both isozymes prefer long carbon chains. For all secondary alcohols studied, the K_m and k_{cat} values for $\beta_2\beta_2$ are much higher than those for $\gamma_1\gamma_1$, i.e., 25- to 360-fold and 6- to 16-fold, respectively. However, for each isozyme the values of k_{cat} for different alcohols are essentially invariant. For a given chain length the isozymes favor primary over secondary alcohols. Secondary alcohol oxidation by the heterodimers $\alpha\beta_2$ and $\beta_2\gamma_1$ follows non Michaelis–Menten kinetics, which can be interpreted in terms of a high-affinity and a low-affinity enzyme component. Values of K_m and k_{cat} estimated from the Eadie–Hofstee plot curves for both of these isozymes indicate that the high-affinity activity of these heterodimers is contributed by the α and γ_1 subunits, respectively, while the low-affinity activity is largely due to the β_2 subunit. These results suggest that the subunits of β_2-containing heterodimeric ADH act independently and noncooperatively. The observed difference between the β_2 chain and the α and γ_1 chains in their binding affinities toward secondary alcohols can be related to structural differences in the substrate-binding sites of these isozymes.

Alcohol dehydrogenase (alcohol:NAD$^+$ oxidoreductase, EC 1.1.1.1) (ADH)[1] is the principal enzyme that catalyzes the metabolic oxidation of alcohols in mammals. In man, ADH exists mainly in the liver and consists of a large number of isozymes. The number and amount of ADH isozymes found in human liver vary, seemingly dependent upon the genetic background, age, and state of health of the individual as well as time of storage of the tissue [for a review see Vallee and Bazzone (1983)].

Since Vallee and co-workers first undertook the isolation of human liver ADH (von Wartburg et al., 1964; Blair & Vallee, 1966), more than 20 different ADH isozymes have been identified, all of which are dimers composed of two 40-kDa subunits and four atoms of zinc (Vallee & Bazzone, 1983). While they share many physical and chemical properties, the human ADH isozymes have been differentiated into three distinct classes by Strydom and Vallee (1982) on the basis of electrophoretic mobility and inhibition by pyrazole. Class I ADH includes those forms composed of the α, β, γ_1, and γ_2 subunits described by the genetic model of Smith et al. (1971a,b, 1972). These isozymes exhibit isoelectric points ranging from pH 9 to 11 and are all effectively inhibited by 4-methylpyrazole (Li & Theorell, 1969). Class II (π) ADH has an isoelectric point of 8.6 and is relatively insensitive to 4-methylpyrazole inhibition (Li & Magnes, 1975; Li et al., 1977). Human class III (χ) ADH is characterized by a low isoelectric point, 6.4, and almost complete insensitivity to 4-methylpyrazole inhibition (Parês & Vallee, 1981; Wagner et al., 1984).

The substrate specificity of eight Caucasian class I ADH isozymes ($\alpha\beta_1$, $\alpha\gamma_1$, $\alpha\gamma_2$, $\beta_1\beta_1$, $\beta_1\gamma_1$, $\beta_1\gamma_2$, $\gamma_1\gamma_1$, and $\gamma_2\gamma_2$) has been studied by using methanol, ethanol, ethylene glycol, benzyl alcohol, octanol, cyclohexanol, and 16-hydroxyhexadecanoic acid (Wagner et al., 1983). The K_m and k_{cat} values indicated that, in general, alcohols with bulky substituents are better substrates for all of the isozymes examined. However, for a given alcohol, values of K_m and k_{cat} for different isozymes generally vary only slightly. One isozyme, $\beta_1\beta_1$, was exceptional in having both very low k_{cat} values toward all substrates studied and a particularly high K_m value toward cyclohexanol. Importantly, the experimentally determined kinetic parameters found for the β_1-containing heterodimers studied were not those predicted from the values for the constituent homodimers. This led to the conclusion that

† This work was supported by grants from The Chinese University of Hong Kong and The Endowment for Research in Human Biology, Inc., with funds provided by Joseph E. Seagram and Sons, Inc., through the Samuel Bronfman Foundation, Inc.

[1] Abbreviations: ADH, alcohol dehydrogenase; *ADH*, alcohol dehydrogenase locus; CapGapp, 4-[3-[*N*-(6-aminocaproyl)amino]propyl]-pyrazole; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PMS, phenazine methosulfate; AMP, adenosine 5′-monophosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/87/0426-5726$01.50/0

subunit interaction endows each of these heterodimeric isozymes with distinct kinetic characteristics.

The subunits composing the $\beta_1\beta_1$ isozyme are produced by the ADH_2^1 allele at the ADH_2 gene locus. A variant form of this subunit, β_2, has been identified in the livers of Japanese and Chinese individuals (Stamatoyannopoulos et al., 1975; Harada et al., 1980) among others, and it has been postulated that this is produced at the ADH_2^2 allele (Smith et al., 1973).[2]

Kinetic studies of ADH homo- and heterodimers containing the β_2 subunit with ethanol and acetaldehyde have demonstrated that it differs significantly from the β_1 subunit. In particular, its V_{max} for ethanol oxidation at pH 7.5 is much higher than that for other homodimers, while that for $\beta_1\beta_1$ is much lower (Yin et al., 1984). Moreover, the kinetic properties of the β_2-containing heterodimers were consistent with the interpretation that the two subunits act independently. We were therefore prompted to gain further insight regarding the β_2 form of ADH and its relationship to the metabolism of ethanol in Orientals by examining a range of primary and secondary alcohols as substrates to determine the kinetic behavior of this atypical variant. These studies have been facilitated greatly by the ability to identify the subunit composition of the individual isozymes by a method of electrophoresis in urea–polyacrylamide gels developed recently (Keung et al., 1985).

Materials and Methods

NAD^+ (grades III and AA-1), Tris, ammonia-free glycine, PMS, MTT, and preswollen microgranular form ion-exchange resins were purchased from Sigma Chemical Co., St. Louis, MO. All alcohol substrates were of reagent grade[3] and were purchased from E. Merck, Darmstadt.

Human liver specimens were obtained at post-mortem examination from apparently healthy individuals who had died suddenly. Specimens were stored at −65 °C before use. The phenotypes of the livers were determined by total activity of the liver and the pH–rate profile for ethanol oxidation (Harada et al., 1978).

Purification of Class I ADH Isozymes. Class I ADH isozymes were purified essentially according to the procedure of Wagner et al. (1983). The pH of all solutions was adjusted at 4 °C. In one preparation, 100 g of liver was homogenized in 150 mL of distilled water and then centrifuged at 30000*g* for 45 min. The supernatant was dialyzed at 4 °C against 10 mM Tris-HCl buffer, pH 7.9, for 18 h and was then loaded onto a DEAE-cellulose column (13 × 8 cm) equilibrated with the dialyzing buffer. The column was eluted with the same buffer, and fractions with ADH activity were pooled and concentrated by ultrafiltration. The concentrated enzyme was applied to a 4-[3-[*N*-(6-aminocaproyl)amino]propyl]pyrazole (CapGapp)–Sepharose affinity column (2.5 × 20 cm) in 50 mM Tris–phosphate and 1 mM NAD^+, pH 9.0 (Lange & Vallee, 1976). After all the unbound protein was washed off, bound enzyme was eluted by 0.5 M ethanol in 50 mM Tris–phosphate buffer, pH 9.0. The unresolved mixture of class I isozymes was then concentrated and dialyzed against 5 mM Tris-HCl, pH 7.7, containing 1 mM NAD^+. The dialyzed sample was loaded onto a CM-cellulose column (2.5 × 60 cm) equilibrated with 5 mM Tris-HCl, pH 7.7, containing 1 mM NAD^+. Elution was carried out with 600 mL of equilibrating buffer followed by a 3.5-L linear gradient of 0–30 mM NaCl in the same buffer. Fractions were assayed for ADH activity and were pooled and concentrated accordingly. Rechromatography on a smaller CM-cellulose column (2.5 × 30 cm) in 5 mM Tris-HCl and 1 mM NAD^+, at different pHs depending on isozyme, was sometimes necessary to obtain pure isozymes.

Enzyme and Protein Assay. The activity of ADH was measured spectrophotometrically by following the absorbance increase at 340 nm in an assay medium containing 0.1 M glycine–NaOH buffer at the specified pH, 2.4 mM NAD^+, and an alcohol substrate. Assays to monitor column eluates were performed with 40 mM ethanol at either pH 9.0 or 10.0, depending on the phenotype of the liver. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of NADH/min at 25 °C. All enzyme assays were performed at 25 °C with a Varian Model 210 spectrophotometer. Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Electrophoretic Procedures. Starch gel electrophoresis was performed at pH 8.2 at 720 V for 5 h according to the method of Bosron et al. (1979a). The ADH isozymes were located by activity staining in 200 mL of 50 mM Tris-HCl buffer, pH 8.5, containing 0.6 mM MTT, 0.26 mM PMS, 1.2 mM NAD^+, and 0.1 M ethanol.

The class I ADH isozymes were identified by determining their subunit compositions by electrophoresis in polyacrylamide gels containing 7 M urea according to the procedure of Keung et al. (1985). Electrophoresis was run at neutral pH by using a discontinuous buffer system, histidine–MOPS, where K^+ and histidine were the leading and trailing ions, respectively. Electrophoresis was performed at 10 °C in a constant-current mode, starting at 10 mA for 30 min and then at 20 mA for 6 h. The gels were stained with 0.05% (w/v) Coomassie Blue in 10% acetic acid and 25% 2-propanol for 1 h and destained with 10% acetic acid and 25% methanol.

Results

Purification of Class I ADH Isozymes. The ADH activity of most of the Chinese livers processed in our laboratory is approximately 20 units per gram of wet liver tissue when assayed at pH 9.0, much higher than that of Caucasian livers under the same circumstances, i.e., about 1 unit per gram of liver. After partial purification on DEAE-cellulose, the class I ADH isozymes elute from the CapGapp–Sepharose column as a group with 0.5 M ethanol. Electrophoresis on starch gel shows that this fraction is devoid of both class II (Bosron et al., 1977) and class III (Wagner et al., 1984) isozymes. Sodium dodecyl sulfate gel electrophoresis reveals only a single band migrating with an apparent molecular weight of 41 000.

Chromatography on CM-cellulose with a linear gradient from 0 to 30 mM NaCl resolves the class I ADH isozymes into active components whose number varies from liver to liver. In some cases, pooled fractions are still heterogeneous and require rechromatography on CM-cellulose at pH 8.2 to obtain pure isozymes. In the present study, four β_2-containing class I ADH isozymes, $\alpha\beta_2$, $\beta_2\gamma_1$, $\beta_2\gamma_2$, and $\beta_2\beta_2$, were obtained from the livers of Orientals and judged to be homogeneous by starch gel electrophoresis. The isozymes were identified by electrophoresis in 7 M urea (Keung et al., 1985) (Figure 1). The specific activities of the four isozymes toward ethanol are similar and range from 4.0 to 6.5 units/(mg of protein). Usually very little, if any, $\gamma_1\gamma_1$ isozyme is isolated from such Oriental livers. The $\gamma_1\gamma_1$ isozyme used in the present study

[2] The β_1 isozyme form is found primarily in livers obtained from Caucasians, β_2 is mostly from Orientals, and β_3, also referred to as $\beta_{Indianapolis}$, is present in livers from Black populations.

[3] Both 2-butanol and 2-octanol were used as their racemates. The enantiomeric specificity of human liver has not been described, but it is known that the horse enzyme has a high degree of stereoselectivity.

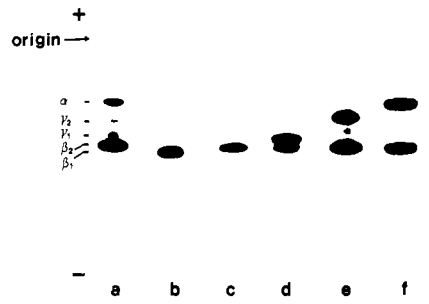

FIGURE 1: Electrophoretic separation of the subunits of purified ADH isozymes in the presence of 7 M urea: (a) unresolved Oriental class I ADH isozymes; (b) $\beta_1\beta_1$; (c) $\beta_2\beta_2$; (d) $\beta_2\gamma_1$; (e) $\beta_2\gamma_2$; (f) $\alpha\beta_2$.

Table I: K_m Values[a] (μM) for Oriental Class I ADH Isozymes toward Primary Alcohols

substrate	isozyme $\alpha\beta_2$	$\beta_2\gamma_1$	$\beta_2\gamma_2$	$\beta_2\beta_2$
methanol	61000	94000	21000	39000
ethanol	3400	3700	3100	3200
1-propanol	1800	1600	1600	1600
1-butanol	510	450	470	410
1-pentanol	420	400	420	410
1-hexanol	96	120	100	85
1-octanol	26	47	38	26

[a] Enzyme activity was assayed at 25 °C in 0.1 M glycine–NaOH buffer, pH 9.0, containing 2.4 mM NAD^+ and various concentrations of alcohol. Whenever possible, the substrate concentrations examined ranged from at least 5-fold below to at least 5-fold above K_m. The kinetic parameters were estimated by Lineweaver–Burk plots.

Table II: k_{cat} Values[a] (min^{-1}) for Oriental Class I ADH Isozymes toward Primary Alcohols

substrate	isozyme $\alpha\beta_2$	$\beta_2\gamma_1$	$\beta_2\gamma_2$	$\beta_2\beta_2$
methanol	15	20	10	18
ethanol	470	460	410	670
1-propanol	490	400	400	700
1-butanol	480	430	380	620
1-pentanol	490	420	380	640
1-hexanol	420	470	340	570
1-octanol	340	300	290	430

[a] See Table I for conditions.

was isolated from a Caucasian liver by similar procedures and had a specific activity of 0.84 unit/(mg of protein) at pH 9.0.

Oxidation of Primary Aliphatic Alcohols by the Purified Oriental Class I ADH Isozymes. The steady-state kinetics of the four purified Oriental ADH isozymes, $\alpha\beta_2$, $\beta_2\gamma_1$, $\beta_2\gamma_2$, and $\beta_2\beta_2$, were studied at pH 9.0 with a series of primary aliphatic alcohols as substrates. In each case, linear $1/v$ vs. $1/S$ plots were obtained over the range of substrate concentration examined. The values of K_m determined from such plots are shown in Table I. For different isozymes, values of K_m vary 3600-fold, e.g., from 26 μM for $\alpha\beta_2$ and $\beta_2\beta_2$ acting on 1-octanol to 94000 μM for $\beta_2\gamma_1$ with methanol as substrate. However, for any given alcohol, the variation between the four isozymes is relatively small, i.e., less than a factor of 2 except for methanol. For each of the isozymes K_m decreases as the chain length of the alcohol substrate increases.

In contrast to the marked substrate dependence of K_m, the values of k_{cat} (Table II) for the oxidation of primary alcohols, except methanol, vary less than 3-fold, i.e., from 290 min^{-1} for $\beta_2\gamma_2$ acting on 1-octanol to 700 min^{-1} for $\beta_2\beta_2$ acting on 1-propanol. The difference in k_{cat} values among the four isozymes toward any given alcohol is even smaller, at most a factor of 2, with $\beta_2\beta_2$ always the highest. Notably, k_{cat} values for methanol oxidation by all these isozymes are ~20-fold

Table III: k_{cat}/K_m Values[a] ($\times 10^{-5}$ M^{-1} min^{-1}) for Oriental Class I ADH Isozymes toward Primary Alcohols

substrate	isozyme $\alpha\beta_2$	$\beta_2\gamma_1$	$\beta_2\gamma_2$	$\beta_2\beta_2$
methanol	0.0025	0.0021	0.0048	0.0046
ethanol	1.4	1.2	1.3	2.1
1-propanol	2.7	2.5	2.5	4.4
1-butanol	9.4	9.6	8.1	15.1
1-pentanol	11.7	10.5	9.0	15.6
1-hexanol	43.8	39.2	34.0	67.1
1-octanol	130.8	63.8	76.0	165.0

[a] See Table I for conditions.

Table IV: K_m Values[a] (μM) for Human Liver ADH Isozymes toward Secondary Alcohols

substrate	$\beta_2\beta_2$	$\gamma_1\gamma_1$	$\alpha\beta_2$ low	$\alpha\beta_2$ high	$\beta_2\gamma_1$ low	$\beta_2\gamma_1$ high
2-propanol	180000	500	190000	200	120000	690
2-butanol[b]	71000	320	80200	45	99000	330
2-octanol[b]	1300	52	720	23	720	34

[a] The assay conditions are similar to those described in Table I. The kinetic parameters for the low-affinity (low) component and the high-affinity (high) component of the heterodimers were estimated by Eadie–Hofstee plots without making any correction for the contribution of the high-affinity system to the low-affinity system and vice versa. [b] Both 2-butanol and 2-octanol were used as their racemates.

Table V: k_{cat} Values[a] (min^{-1}) for Human Liver ADH Isozymes toward Secondary Alcohols

substrate	$\beta_2\beta_2$	$\gamma_1\gamma_1$	$\alpha\beta_2$ low	$\alpha\beta_2$ high	$\beta_2\gamma_1$ low	$\beta_2\gamma_1$ high
2-propanol	280	18	150	21	150	9
2-butanol	280	30	150	27	160	17
2-octanol	270	44	120	40	170	29

[a] See Table IV for details.

Table VI: k_{cat}/K_m Values[a] ($\times 10^{-5}$ M^{-1} min^{-1}) for Human Liver ADH Isozymes toward Secondary Alcohols

substrate	$\beta_2\beta_2$	$\gamma_1\gamma_1$	$\alpha\beta_2$ low	$\alpha\beta_2$ high	$\beta_2\gamma_1$ low	$\beta_2\gamma_1$ high
2-propanol	0.016	0.36	0.0079	1.1	0.013	0.13
2-butanol	0.039	0.94	0.019	6.0	0.016	0.52
2-octanol	2.10	8.50	1.7	17.0	2.4	8.50

[a] See Table IV for details.

lower than those obtained for the oxidation of other alcohols. Table III shows the values of k_{cat}/K_m that best reflect the specificities of the isozymes. In general, $\beta_2\beta_2$ exhibits the highest k_{cat}/K_m toward all substrates examined, while among the various alcohols, the longer the chain length the higher the k_{cat}/K_m. Importantly, the k_{cat}/K_m values for ethanol for all of the isozymes studied are lower than for any other alcohol except methanol (Table III).

The normalized pH–activity profiles of $\alpha\beta_2$, $\beta_2\beta_2$, $\beta_2\gamma_1$, and $\beta_2\gamma_2$ with ethanol as substrate are virtually superimposable (Figure 2). Maximum activity occurs at pH 9.0 instead of pH 10–10.5 as is seen with $\alpha\alpha$, $\gamma_1\gamma_1$, and $\gamma_2\gamma_2$ (Bosron et al., 1979b, 1983).

Oxidation of Secondary Aliphatic Alcohols by Human Class I ADH Isozymes. The steady-state kinetics for the oxidation of secondary alcohols by $\alpha\beta_2$, $\beta_2\gamma_1$, $\beta_2\beta_2$, and $\gamma_1\gamma_1$ were studied with 2-propanol, 2-butanol, and 2-octanol. The homodimeric ADH isozymes $\beta_2\beta_2$ and $\gamma_1\gamma_1$ follow apparent Michaelis–Menten kinetics, and their kinetic parameters are listed in Tables IV–VI. Values of K_m and k_{cat}/K_m again indicate that longer chain alcohols are generally better substrates for both isozymes. Between the two isozymes, values

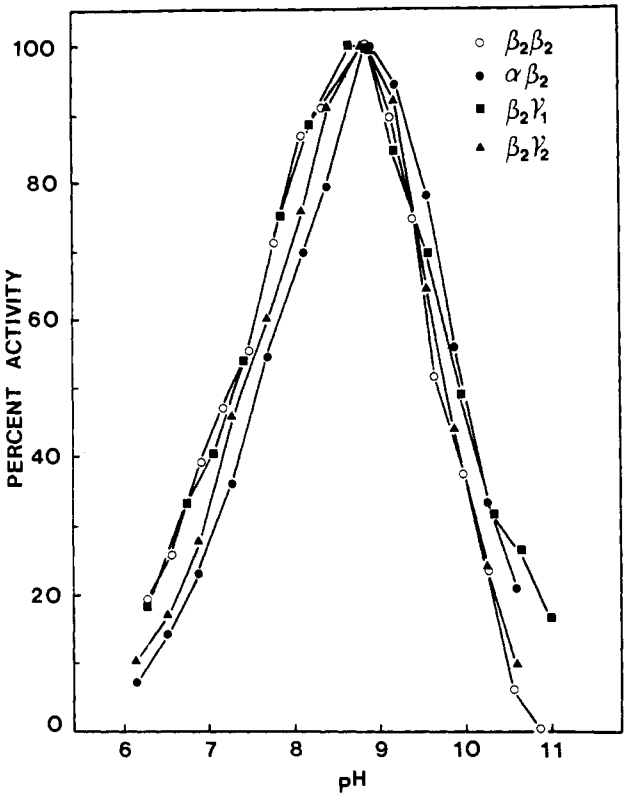

FIGURE 2: pH–activity profiles of $\beta_2\beta_2$, $\alpha\beta_2$, $\beta_2\gamma_1$, and $\beta_2\gamma_2$. Reaction rates were assayed with 2.4 mM NAD^+ and 40 mM ethanol, in 0.1 M glycine–NaOH buffer (pH 8.3–11.0) or 0.1 M Hepes buffer (pH 6.0–8.8).

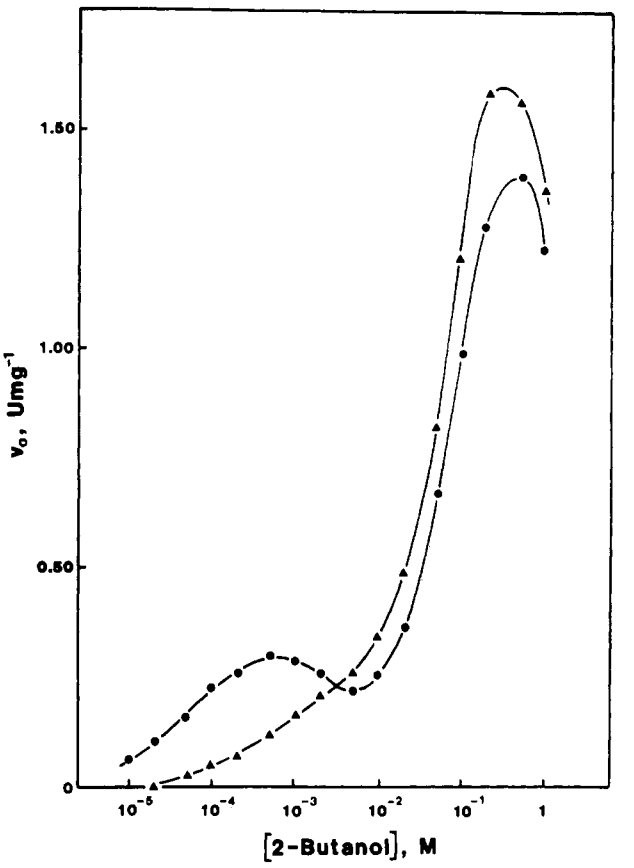

FIGURE 3: Kinetic response of heterodimeric ADH isozymes $\alpha\beta_2$ (●) and $\beta_2\gamma_1$ (▲) to 2-butanol concentration. The enzymes were assayed in 0.1 M glycine–NaOH buffer, pH 9.0, containing 2.4 mM NAD^+.

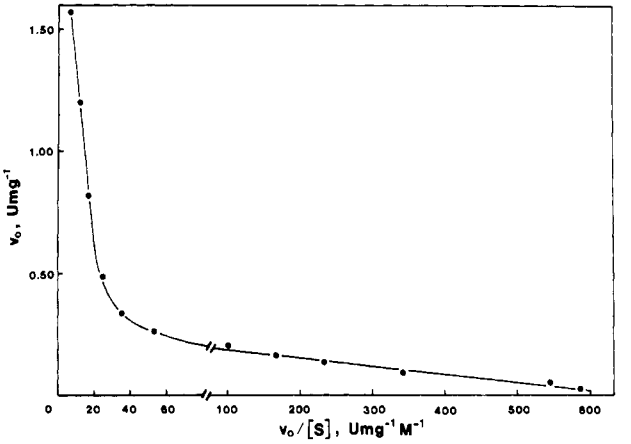

FIGURE 4: Eadie–Hofstee plot of the kinetic data for 2-butanol oxidation catalyzed by $\beta_2\gamma_1$, taken from Figure 3. The kinetic constants derived from the plot are listed in Tables IV–VI.

of K_m measured for any given alcohol differ significantly, and the difference increases as the chain length of the substrate decreases, from 2-octanol, 25-fold, to 2-propanol, 360-fold. The k_{cat} values for $\beta_2\beta_2$ are 6–15 times greater than those for $\gamma_1\gamma_1$, depending on the substrate. For $\beta_2\beta_2$, k_{cat} values for the oxidation of all secondary alcohols are essentially the same, whereas those for $\gamma_1\gamma_1$ vary, but not greatly.

The kinetics for the heterodimers $\alpha\beta_2$ and $\beta_2\gamma_1$ do not follow simple Michaelis–Menten kinetics. This is illustrated by the substrate concentration dependence for the oxidation of 2-butanol by the heterodimers, as shown in Figure 3. Thus, for $\alpha\beta_2$, activity first increases as the concentration of 2-butanol increases from 10 to 500 μM. At higher concentrations of 2-butanol, activity decreases but then again increases sharply to reach a peak at 0.5 M. Biphasic kinetics are obvious for $\beta_2\gamma_1$ from the analysis of the data by Eadie–Hofstee plots, which show that the kinetics of this heterodimer can be resolved into high- and low-affinity components (Figure 4). Analogous results were also observed for the other secondary alcohols (not shown). The values of K_m and k_{cat} for the two components of the heterodimers estimated by Eadie–Hofstee analysis are included in Tables IV–VI. Values of K_m and k_{cat}/K_m indicate that both components prefer substrates with a long carbon chain. Furthermore, the K_m values of the high-affinity component are similar to those measured for $\gamma_1\gamma_1$, while the K_m values of the low-affinity component are in the same range as those for $\beta_2\beta_2$. Moreover, it should be noted that the k_{cat} values of the low-affinity component of $\alpha\beta_2$ and $\beta_2\gamma_1$ are about half that measured for $\beta_2\beta_2$, while the k_{cat} value of the high-affinity component of $\beta_2\gamma_1$ is about half that measured for $\gamma_1\gamma_1$.

In order to examine the reaction mechanism of $\beta_2\beta_2$, kinetic studies were carried out with 2-propanol as substrate as a function of the concentration of product, NADH. As shown in Figure 5, NADH is a competitive inhibitor of NAD^+ under these conditions, consistent with the ordered, sequential mechanism that has been proposed for the horse and other human liver ADH isozymes (Wratten & Cleland, 1963; Bosron et al., 1979b, 1983; Yin et al., 1984). Similar results were obtained with both saturating, 2 M (Figure 5), and limiting, 100 mM (not shown), concentrations of 2-propanol. The other product, acetone, is a very poor substrate for $\beta_2\beta_2$ and, in fact, acts as a mixed-uncompetitive inhibitor of NAD^+.

The effects of pH on the high- and low-affinity activities of the heterodimers $\alpha\beta_2$ and $\beta_2\gamma_1$ were obtained at both low and high substrate concentrations, respectively, and compared with the effect on pH on secondary alcohol oxidation by $\beta_2\beta_2$ and $\gamma_1\gamma_1$. As shown in Table VII, the ratios of the activities

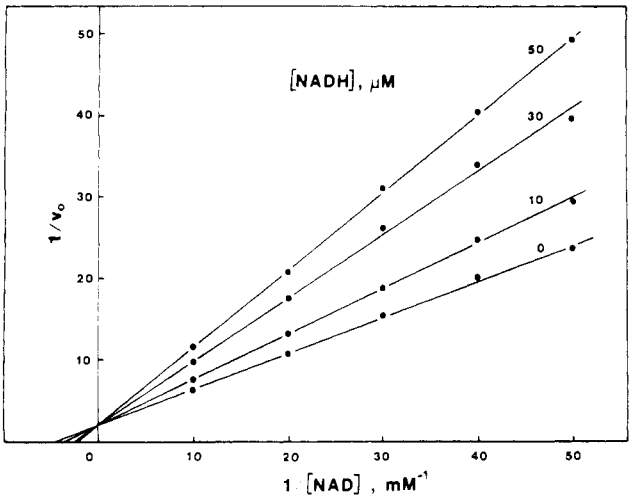

FIGURE 5: Product inhibition of $\beta_2\beta_2$ by NADH. Enzyme activity was determined in 0.1 M glycine–NaOH buffer, pH 9.0, at 25 °C with 2 M 2-propanol.

Table VII: Effect of pH on Secondary Alcohol Oxidation by Human Liver ADH Isozymes

substrate	concn (mM)	activity ratio (pH 10.5/pH 9.5) $\gamma_1\gamma_1$	$\beta_2\beta_2$	$\alpha\beta_2$	$\beta_2\gamma_1$
2-propanol	2	1.49	*a*	1.37	1.04
	1000	1.63	0.67	0.86	0.98
2-butanol	0.5	1.58	*a*	1.30	1.39
	200	1.62	0.60	0.81	0.79
2-octanol	0.1	1.55	0.70	1.11	1.12
	5	1.66	0.81	0.96	0.75

[a] No appreciable activity can be detected at these concentrations at either pH 10.5 or pH 9.5.

of $\gamma_1\gamma_1$ at pH 10.5 to those at pH 9.5 are always greater than 1, whereas the corresponding ratios determined for $\beta_2\beta_2$ are always less than 1. Similar to $\gamma_1\gamma_1$, the high-affinity activity of either $\alpha\beta_2$ or $\beta_2\gamma_1$, measured at the lower substrate concentration, is also more active at pH 10.5, while the low-affinity component, like $\beta_2\beta_2$, is more active at pH 9.5.

Oxidation of 2-Substituted Primary Alcohols by Human Class I ADH Isozymes. The steady-state kinetics of $\alpha\beta_2$, $\beta_2\beta_2$, $\beta_2\gamma_1$, and $\gamma_1\gamma_1$ were also studied at pH 9.0 by using 2-substituted primary alcohols as substrates. The 2-substituted primary alcohols differ from the secondary alcohols by having a bulky substituent one carbon atom farther away from the hydroxyl function. Table VIII shows the K_m values for the oxidation of benzyl alcohol and 2-ethyl-1-hexanol by the various ADH isozymes. The differences between the K_m values of $\beta_2\beta_2$ and $\gamma_1\gamma_1$ and presumably $\alpha\alpha$ and $\gamma_2\gamma_2$ toward these 2-substituted primary alcohols are also large enough to result in biphasic kinetics for β_2-containing heterodimers. This is apparent from the two K_m values obtained for $\beta_2\gamma_1$, which resemble those of $\beta_2\beta_2$ and $\gamma_1\gamma_1$, respectively (Table VIII).

Table VIII: K_m Values[a] (μM) for Human Liver ADH Isozymes toward 2-Substituted Primary Alcohols

substrate	$\beta_2\beta_2$	$\gamma_1\gamma_1$	$\alpha\beta_2$ low	$\alpha\beta_2$ high	$\beta_2\gamma_1$ low	$\beta_2\gamma_1$ high
benzyl alcohol	1700	7[b]	1800	3	1600	7
2-ethyl-1-hexanol	3760	*c*	1530	4	1390	12

[a] The assay conditions are similar to those described in Table I. K_m values for the low-affinity (low) component and the high-affinity (high) component were estimated by Eadie–Hofstee plots without correcting for the contribution of the high-affinity system to the low-affinity system and vice versa. [b] From Wagner et al. (1983), assayed at pH 10.0. [c] Not determined.

DISCUSSION

Human liver ADH catalyzes the oxidation/reduction of a wide spectrum of substrates including primary and secondary aliphatic and aromatic alcohols and their corresponding aldehydes and ketones (Vallee & Bazzone, 1983). A rather extensive examination of the substrate specificity of the class I ADH isozymes from Caucasian livers using a selected number of substrates representing alcohols of metabolic or toxicological significance was carried out by Wagner et al. (1983). However, similar studies with ADH isozymes containing the variant β_2 chain (Oriental ADHs) are not on record. This paper, therefore, reports the first study of this kind on four Oriental-type class I ADH isozymes, $\alpha\beta_2$, $\beta_2\gamma_1$, $\beta_2\gamma_2$, and $\beta_2\beta_2$, purified from livers of Chinese subjects obtained at autopsy from local hospitals in Hong Kong.

The affinity resin CapGapp–Sepharose (Lange & Vallee, 1976) was employed for the isolation of these isozymes. The unresolved class I isozyme mixture purified from most of the of Chinese livers has a specific activity between 4 and 6 units/(mg of protein), depending on the individual livers. This is about 4–6 times higher than that of the unresolved class I ADH isozymes isolated from Caucasian livers and is largely due to the fact that the β_2 subunit is intrinsically more active than the β_1 subunit. Chromatography on CM-cellulose at pH 7.7 is usually sufficient to resolve the class I isozymes, which constitute over 90% of the total ADH activity of the original liver extract. Rechromatography on CM-cellulose at a different pH, generally pH 8.2, yields electrophoretically homogeneous isozymes.

The turnover number for ethanol oxidation by the $\beta_2\beta_2$ homodimer isolated from different livers ranged from 560 to 880 min^{-1}. These values are comparable to those reported by Okuda and Okuda (1983), 848 min^{-1}, but are consistently lower than that estimated for the $\beta_2\beta_2$ isozyme, ~1520 min^{-1}, isolated by Yin et al. (1984).

It has been pointed out that liver specimens from Japanese subjects contain β_2 isozymes which can be classified into two phenotypes, the homozygous ADH_2 2-2 and the heterozygous ADH_2 2-1 phenotypes (Yin et al., 1984). With the ADH_2 2-2 livers, $\beta_2\beta_2$ is the last activity peak to elute from CM-cellulose, and it migrates as a single band on isoelectric focusing. In contrast, the last activity peak of the ADH_2 2-1 livers exhibits three bands on isoelectric focusing corresponding to $\beta_1\beta_1$, $\beta_1\beta_2$, and $\beta_2\beta_2$. Since $\beta_1\beta_1$ is the least active ADH isozyme of all ADH_2s known, the lower turnover number for ethanol observed for the $\beta_2\beta_2$ isozymes isolated earlier (Okuda & Okuda, 1983) might have been due to mixtures of $\beta_1\beta_1$, $\beta_1\beta_2$, and $\beta_2\beta_2$ that were not resolved by CM-cellulose and perhaps less well resolved on starch gel electrophoresis, as is often the case. In the present study, the subunit compositions of the individual isozymes were identified by electrophoresis of urea-dissociated enzymes on polyacrylamide gels containing 7 M urea in a neutral, discontinuous histidine–Mops buffer system (Keung et al., 1985). This method has been shown to resolve all subunit chains found in Caucasian class I and class II ADH isozymes—α, β_1, γ_1, γ_2, and π—and to provide a simple, reproducible method for identifying human class I ADHs. It has been extended in this system to the identification of the Oriental-type (β_2) ADH isozymes. Careful analysis of gels (Figure 1) indicates that, although the β_1, β_2, and γ_1 chains are not separated as well as the α and γ_2 chains, they do have different electrophoretic mobilities, with β_1 migrating fastest, followed by β_2, γ_1, γ_2, and α. The preparation was chromatographed on an AMP–hexane–agarose column according to

the procedure of Yin et al. (1984), which established that the $\beta_2\beta_2$ used in the present study did not contain any β_1-containing isozyme. Only one activity peak was eluted at a gradient NADH concentration similar to that reported for $\beta_2\beta_2$ (results not shown). Furthermore, isoelectric focusing of the $\beta_2\beta_2$ preparation failed to detect any β_1 isozyme. Thus, the difference in activity observed for the $\beta_2\beta_2$ isolated here and that of Yin et al. (1984) may be due either to the existence of an intrinsic structural difference between the two preparations, obtained from livers of Japanese and Chinese subjects, respectively, or to artifacts of the purification procedures.

The kinetic parameters obtained for the individual β_2-containing isozymes are virtually invariant for the same isozymes isolated from at least three different livers. Activities ranged from 410 to 540, 430 to 510, 390 to 420, and 560 to 880 min^{-1} for $\alpha\beta_2$, $\beta_2\gamma_1$, $\beta_2\gamma_2$, and $\beta_2\beta_2$, respectively. As with the Caucasian-type ADHs, the substrate specificity of the β_2-type isozymes was also apparent from the value of K_m. With all primary alcohols from ethanol to 1-octanol the k_{cat} values varied less than 2-fold, whereas the K_m values varied more than 100-fold. However, the K_m values for the oxidation of any single primary alcohol—except methanol—by all four β_2-containing isozymes are essentially identical, suggesting that the α, β_2, γ_1, and γ_2 isozymes share similar substrate specificities toward primary alcohols. Importantly, although ethanol might be thought to be the principal physiological substrate for ADH, it is actually oxidized quite poorly. In fact, depending on the particular isozyme, it is oxidized from 50- to 90-fold less effectively than 1-octanol.

The activities observed for the heterodimers $\alpha\beta_2$, $\beta_2\gamma_1$, and $\beta_2\gamma_2$ are contributed largely by the β_2 subunit, which is common to all four isozymes examined. The predominance of the β_2 subunit in these isozymes is also implied by the observation that, although the pH–activity profiles for $\alpha\alpha$, $\gamma_2\gamma_2$, and $\gamma_1\gamma_1$ are maximal at 10.0–10.5 (Bosron et al., 1979b, 1983), those for the three β_2-containing heterodimers $\alpha\beta_2$, $\beta_2\gamma_1$, and $\beta_2\gamma_2$ are more like that of $\beta_2\beta_2$ (Figure 2). Moreover, for the oxidation of any primary alcohol, the k_{cat} value for $\beta_2\beta_2$ is, in general, 40–70% higher than those measured for $\alpha\beta_2$, $\beta_2\gamma_1$, or $\beta_2\gamma_2$. Since the specific activities for $\alpha\alpha$, $\gamma_1\gamma_1$, and $\gamma_2\gamma_2$ are about 80% lower than that of $\beta_2\beta_2$, the simplest explanation for this observation would be that the individual subunits contribute independently to the total activity of the dimeric enzymes.

When primary alcohols are the substrates, kinetic properties of the α, γ_1, and γ_2 subunits in the heterodimers $\alpha\beta_2$, $\beta_2\gamma_1$, and $\beta_2\gamma_2$, respectively, cannot be resolved from that of the β_2 subunit because they all have similar K_m values. However, the wide difference between the K_m values of $\beta_2\beta_2$ and $\gamma_1\gamma_1$ and presumably $\alpha\alpha$ and $\gamma_2\gamma_2$ toward secondary alcohols makes it possible to detect the contributions of the α, γ_1, and γ_2 subunits in the corresponding heterodimers. All of the secondary alcohols examined bind weakly to $\beta_2\beta_2$ and display non Michaelis–Menten kinetics with β_2-containing heterodimers. In particular, $\beta_2\beta_2$ oxidizes cyclohexanol with an extremely high K_m value, 0.11 M, as compared with those of isozymes containing α, γ_1, and γ_2 chains of 5 to 80 μM (Fong & Keung, 1987). We have taken advantage of this marked difference in K_m to establish the contributions of the individual subunits to the overall activity of β_2-containing heterodimers. That study substantiates the present supposition that in such heterodimers the individual subunits act independently.

Sequence analysis of the active-site peptides of human ADH isozymes has shown that in this region the α, β, and γ polypeptide chains are structurally distinct. Replacements occur at positions that influence (A) coenzyme binding, e.g., position 47 is Gly in α, Arg in β_1 and γ, and His in β_2 and (B) substrate binding, e.g., position 48 is Thr in α and β and Ser in γ and position 93 is Phe in β and γ and Ala in α (Hempel et al., 1984; Bühler et al., 1984a–c,; Jörnvall et al., 1984; Deuster et al., 1984; Ikuta et al., 1985; Hempel et al., 1985).

Recently, three-dimensional models of the subunits of human class I ADH have been generated on the basis of homologous horse liver enzyme by using computer graphics (Eklund et al., 1987). These models clearly indicate why both $\beta_1\beta_1$ and $\beta_2\beta_2$, which differ only in position 47—arginine in $\beta_1\beta_1$ and histidine in $\beta_2\beta_2$—should exhibit low binding affinity for cyclohexanol and for secondary alcohols in general: their substrate-binding pocket includes a threonine at position 48 and a phenylalanine at position 93. The methyl group of Thr-48 is in the inner part of the substrate pocket, close to the active-site zinc atom, and adjacent to Phe-93. This literally creates a bottleneck at the entrance to the pocket, impeding the approach of substrates with bulky α-carbon substituents. To a lesser extent this also impedes the approach of substrates with bulky β-carbon substituents (Table VIII). Molecular modeling experiments (Horjales & Brändén, 1985; Eklund et al., 1987) also indicate that in order to accommodate the cyclohexanol molecule Phe-93 of the β_1 subunit—and presumably also the β_2 subunit—would have to change their side-chain torsion angles by 15° and 23°, respectively, from the angles in the E-type subunit of the horse enzyme. This is more than twice the change of torsion angles for Phe-93 in the γ subunits. While the α subunit also has a threonine at position 48, the other side of its binding pocket is much wider since position 93 is Ala rather than Phe. The absence of the phenyl side chain apparently allows the α subunit to accommodate cyclohexanol more readily.

Values of k_{cat} for $\beta_2\beta_2$ assayed with a series of primary aliphatic alcohols under a given set of conditions are relatively invariant, 430–700 min^{-1}, with the exception of methanol, 18 min^{-1}. For horse liver ADH, the rate of methanol oxidation is known to be limited by the catalytic step (Brooks & Shore, 1971). The constancy of the k_{cat} values for the human ADH isozymes toward primary aliphatic alcohols, C_2 and longer, probably reflects the dissociation of NADH, which is the usual rate-limiting step (Dalziel & Dickerson, 1966; Wratten & Cleland, 1963; Dworschack & Plapp, 1977), whereas the k_{cat} for methanol reflects the catalytic step. Extending this rationale to the lower but qualitatively invariant k_{cat} values for $\beta_2\beta_2$ toward secondary aliphatic alcohols (260–280 min^{-1}) could also reflect a mechanism in which the rate is not limited by NADH dissociation. Accordingly, the reaction mechanism of purified $\beta_2\beta_2$ was examined by steady-state kinetic studies at pH 9.0 using 2-propanol as substrate. NADH was found to be a competitive inhibitor of NAD^+ at both limiting, 100 mM, and saturating, 2 M, concentrations of 2-propanol (Figure 5), while under these same conditions acetone behaves as a mixed-uncompetitive inhibitor of NAD^+ (data not shown). Such inhibition patterns are consistent with the ordered, sequential mechanism proposed for the horse and the other human liver ADH isozymes (Wratten & Cleland, 1963; Bosron et al., 1979b, 1983; Yin et al., 1984). The rate constant for NADH dissociation from $\beta_2\beta_2$ was reported to be approximately equal to the k_{cat} for ethanol oxidation. The lower k_{cat} values observed for the oxidation of secondary alcohols may therefore reflect a rate-determining step other than NADH dissociation.

Registry No. ADH, 9031-72-5; NADH, 58-68-4; methanol, 67-56-1; ethanol, 64-17-5; 1-propanol, 71-23-8; 1-butanol, 71-36-3; 1-

pentanol, 71-41-0; 1-hexanol, 111-27-3; 1-octanol, 111-87-5; 2-propanol, 67-63-0; 2-butanol, 78-92-2; 2-octanol, 123-96-6; benzyl alcohol, 100-51-6; 2-ethyl-1-hexanol, 104-76-7.

REFERENCES

Blair, A. H., & Vallee, B. L. (1966) *Biochemistry 5*, 2026–2034.

Bosron, W. F., Li, T.-K., Lange, L. G., Dafeldecker, W. P., & Vallee, B. L. (1977) *Biochem. Biophys. Res. Commun. 74*, 85–91.

Bosron, W. F., Li, T.-K., & Vallee, B. L. (1979a) *Biochem. Biophys. Res. Commun. 91*, 1549–1555.

Bosron, W. F., Li, T.-K., Dafeldecker, W. P., & Vallee, B. L. (1979b) *Biochemistry 18*, 1101–1105.

Bosron, W. F., Magnes, L. J., & Li, T.-K. (1983) *Biochemistry 22*, 1852–1857.

Brooks, R. L., & Shore, J. D. (1971) *Biochemistry 10*, 3855–3858.

Bühler, R., Hempel, J., Kaiser, R., De Zaleuski, C., von Wartburg, J.-P., & Jörnvall, H. (1984a) *Eur. J. Biochem. 145*, 447–453.

Bühler, R., Hempel, J., Kaiser, R., von Wartburg, J.-P., Vallee, B. L., & Jörnvall, H. (1984b) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6320–6324.

Bühler, R., Hempel, J., von Wartburg, J.-P., & Jörnvall, H. (1984c) *FEBS Lett. 173*, 360–366.

Dalziel, K., & Dickerson, F. M. (1966) *Biochem. J. 100*, 34–46.

Deuster, G., Hatfield, G. W., Bühler, R., Hempel, J., Jörnvall, H., & Smith, M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4055–4059.

Dworschack, R. T., & Plapp, B. V. (1977) *Biochemistry 16*, 111–116.

Eklund, H., Horjales, E., Vallee, B. L., & Jörnvall, H. (1987) *Eur. J. Biochem.* (submitted for publication).

Fong, W. P., & Keung, W. M. (1987) *Biochemistry* (following paper in this issue).

Harada, S., Agarwal, D. P., & Goedde, H. W. (1978) *Hum. Genet. 40*, 215–220.

Harada, S., Misawa, S., Agarwal, D. P., & Goedde, W. (1980) *Am. J. Hum. Genet. 32*, 8–15.

Hempel, J., Bühler, R., Kaiser, R., Holmquist, B., De Zaleuski, C., von Wartburg, J.-P., Vallee, B. L., & Jörnvall, H. (1984) *Eur. J. Biochem. 145*, 437–445.

Hempel, J., Holmquist, B., Fleetwood, L., Kaiser, R., Barros-Söderling, J., Bühler, R., Vallee, B. L., & Jörnvall, H. (1985) *Biochemistry 24*, 5303–5307.

Horjales, E., & Brändén (1985) *J. Biol. Chem. 260*, 15445–15451.

Ikuta, T., Fujiyoshi, T., Kurachi, K., & Yoshida, A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2703–2707.

Jörnvall, H., Hempel, J., Vallee, B. L., Bosron, W. F., & Li, T.-K. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3024–3028.

Keung, W. M., Ditlow, C. C., & Vallee, B. L. (1985) *Anal. Biochem. 151*(1), 92–96.

Lange, L. G., & Vallee, B. L. (1976) *Biochemistry 15*, 4681–4686.

Li, T.-K., & Theorell, H. (1969) *Acta Chem. Scand., Ser. B 23*, 892–902.

Li, T.-K., & Magnes, L. J. (1975) *Biochem. Biophys. Res. Commun. 63*, 202–208.

Li, T.-K., Bosron, W. F., Dafeldecker, W. P., Lange, L. G., & Vallee, B. L. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4378–4381.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Okuda, A., & Okuda, K. (1983) *J. Biol. Chem. 258*, 2899–2905.

Parês, X., & Vallee, B. L. (1981) *Biochem. Biophys. Res. Commun. 98*, 122–130.

Smith, M., Hopkinson, D. A., & Harris, H. (1971a) *Ann. Hum. Genet. 34*, 251–271.

Smith, M., Hopkinson, D. A., & Harris, H. (1971b) *Eur. J. Biochem. 24*, 251–271.

Smith, M., Hopkinson, D. A., & Harris, H. (1972) *Ann. Hum. Genet. 35*, 243–253.

Smith, M., Hopkinson, D. A., & Harris, H. (1973) *Ann. Hum. Genet. 37*, 49–67.

Stamatoyannopoulos, G., Chen, S. H., & Fukui, M. (1975) *Am. J. Hum. Genet. 27*, 789–796.

Strydom, D. S., & Vallee, B. L. (1982) *Anal. Biochem. 123*, 422–429.

Vallee, B. L., & Bazzone, T. J. (1983) in *Isozymes: Current Topics in Biological and Medical Research* (Scandalios, J. G., Rattazzi, M. C., & Whitt, G. S., Eds.) Vol. 8, pp 219–244, Alan R. Liss, New York.

von Wartburg, J. P., Bethune, J. L., & Vallee, B. L. (1964) *Biochemistry 3*, 1775–1782.

Wagner, F. W., Burger, A. R., & Vallee, B. L. (1983) *Biochemistry 22*, 1857–1863.

Wagner, F. W., Parês, X., Holmquist, B., & Vallee, B. L. (1984) *Biochemistry 23*, 2193–2199.

Wratten, C. C., & Cleland, W. W. (1963) *Biochemistry 2*, 935–941.

Yin, S. J., Bosron, W. F., Magnes, L. J., & Li, T.-K. (1984) *Biochemistry 23*, 5847–5853.