Human Liver Alcohol Dehydrogenase: Purification and Kinetic Characterization of the $\beta_2\beta_2$, $\beta_2\beta_1$, $\alpha\beta_2$, and $\beta_2\gamma_1$ "Oriental" Isoenzymes†

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ABSTRACT: Four alcohol dehydrogenase isoenzymes with "atypical" pH optima for ethanol oxidation at 8.8 were isolated from Japanese livers with the homozygous ADH_2 2-2 and the heterozygous ADH_2 2-1 phenotypes. Agarose gel isoelectric focusing patterns after dissociation–recombination of three isoenzymes purified from the homozygous livers indicate that they are $\beta_2\beta_2$, $\alpha\beta_2$, and $\beta_2\gamma_1$. A fourth isoenzyme, purified from livers with the heterozygous phenotype by agarose–hexane–AMP affinity chromatography, was identified as $\beta_2\beta_1$ by dissociation–recombination studies. The kinetic properties of the three heterodimers, $\beta_2\beta_1$, $\alpha\beta_2$, and $\beta_2\gamma_1$, are intermediate between those of the respective homodimers, suggesting that the two subunits act independently. Product inhibition studies indicate that $\beta_2\beta_2$ obeys an ordered sequential mechanism, as do the $\alpha\alpha$, $\beta_1\beta_1$, $\gamma_1\gamma_1$, and $\gamma_2\gamma_2$ homodimers which have the "typical" pH optimum for ethanol oxidation at pH 10.0–10.5. The kinetic constants of $\beta_2\beta_2$ differ substantially from those of the other homodimers. At pH 7.5, the V_{max} for ethanol oxidation of $\beta_2\beta_2$ is 5–40 times higher than that of $\alpha\alpha$, $\beta_1\beta_1$, $\gamma_1\gamma_1$, and $\gamma_2\gamma_2$. The K_m and K_i values of $\beta_2\beta_2$ for NAD^+ and NADH are also considerably higher than those of the other homodimers. The kinetic differences between the "Oriental" $\beta_2\beta_2$ homodimer and other human liver isoenzymes can be explained by the substitution of a weaker base, histidine, in the coenzyme binding site of $\beta_2\beta_2$ for the arginine-47 residue that appears in all other mammalian alcohol dehydrogenases thus far examined [Jörnvall, H., Hempel, J., Vallee, B. L., Bosron, W. F., & Li, T.-K. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3024–3028].

Human liver alcohol dehydrogenase (alcohol:NAD^+ oxidoreductase, EC 1.1.1.1) exhibits multiple molecular forms that can be identified by their mobility on starch gel electrophoresis (Smith et al., 1971; Harada et al., 1978; Bosron & Li, 1981) and with isoelectric focusing in polyacrylamide or agarose gels (Harada et al., 1978; Yin et al., 1984). The presence of variant isoenzymes produced at the polymorphic ADH_2 locus can also be identified in liver homogenate–supernatants by examining pH–activity profiles for ethanol oxidation (von Wartburg & Schürch, 1968; Bosron et al., 1980). For example, the optimum for activity in homogenate–supernatants of livers from white American and European populations is at pH 10.0–10.5, and this has been called the "typical"[1] pH–activity profile (von Wartburg & Schürch, 1968; Smith et al., 1971; Bosron & Li, 1981). These livers contain isoenzymes with α, γ, and β_1 subunits, the latter being produced by the ADH_2^1 allele (Smith et al., 1971). Livers with a variant pH–activity profile characterized by dual optima at pH 7.0 and 10.0 have been found in about 25% of black American population (Bosron et al., 1980). The isoenzymes giving rise to the activity optimum at pH 7.0 contain the $\beta_{Indianapolis}$ (β_{Ind}) subunit that is produced by the variant ADH_2^{Ind} allele (Bosron et al., 1983a). About 20% of livers from a Swiss population exhibit yet another unique pH–activity profile for ethanol oxidation with a single optimum at pH 8.8 (von Wartburg & Schürch, 1968). Greater than 85% of livers from Japanese and Chinese also have this "atypical" pH 8.8 optimum for activity (Fukui & Wakasugi, 1972; Teng et al., 1979; Harada et al., 1980). It has been postulated that this so-called atypical pH–activity profile arises from isoenzymes containing a variant β_2 subunit, produced by the ADH_2^2 allele (Smith et al., 1971).

The characterization of the kinetic and structural properties of the different hetero- and homodimeric isoenzymes containing the β_2 subunits had not been possible until recently because of the lack of methods for their identification and purification. We recently showed that the complex pattern of isoenzymes in Japanese livers with the atypical or "$ADH_{Oriental}$" pH–activity profile can now be divided into the homozygous ADH_2 2-2 and heterozygous ADH_2 2-1 phenotypes by agarose isoelectric focusing of homogenate–supernatants (Yin et al., 1984). Three β_2-containing isoenzymes were purified from a liver with the homozygous phenotype by affinity and cation-exchange chromatography. A fourth isoenzyme containing β_2 subunits was found in livers with the heterozygous phenotype. It was not separable from $\beta_2\beta_2$ and $\beta_1\beta_1$ by conventional cation-exchange chromatography (Berger et al., 1974; Yin et al., 1984), but it was purified by coenzyme affinity chromatography on agarose–hexane–AMP. In this paper, we report the identification of these four β_2-containing isoenzymes by means of dissociation–recombination studies, their specific activities and pH–activity profiles, and the steady-state kinetic constants of the $\beta_2\beta_2$ Oriental isoenzyme.

Experimental Procedures

Alcohol dehydrogenase activity was assayed spectrophotometrically at 340 nm and 25 °C with 33 mM ethanol and 2.4 mM NAD^+ (grade I; Boehringer-Mannheim, Indianapolis, IN) or with 5 mM acetaldehyde and 0.3 mM NADH (grade III; Sigma, St. Louis, MO) in 3 mL of 0.1 M NaP_i[2] (pH 6.0

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[1] The terms "typical" and "atypical" denote that the optimum for alcohol dehydrogenase activity in liver homogenate–supernatants or purified preparations is at pH 10.5 or 8.8, respectively. It has been postulated that livers exhibiting the optimum for ethanol oxidation at pH 8.8 contain isoenzymes with β_2 subunits which are produced by the ADH_2^2 allele at the ADH_2 gene locus. Livers with the pH optimum of 10.5 contain isoenzymes with β_1 subunits produced by ADH_2^1. Livers are the homozygous ADH_2 2-2 phenotype if isoenzymes with β_2 subunits are present. Livers are the homozygous ADH_2 1-1 phenotype if they contain isoenzymes with β_1 subunits. Livers are the heterozygous ADH_2 2-1 phenotype if they contain isoenzymes with both β_2 and β_1 subunits.

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or 7.5), 0.1 M $NaPP_i$ (pH 8.5), or 0.1 M glycine–NaOH (pH 10.0). Enzyme activity units are expressed as micromoles of NADH formed or utilized per minute based on an A_{340} nm of 6.22 mM^{-1} cm^{-1}. The protein concentration of purified isoenzymes was determined with the Folin reagent (Lowry et al., 1951) and that for chromatographic elution profiles was determined with the Bio-Rad protein assay reagent (Bio-Rad, Richmond, CA; Bradford, 1976) with bovine serum albumin as standard.

Alcohol dehydrogenase isoenzymes were identified by high-voltage starch gel electrophoresis at pH 8.2 (Bosron et al., 1979b) and by agarose isoelectric focusing (Yin et al., 1984); gels were stained for ethanol oxidizing activity with thiazolyl blue dye (Bosron et al., 1979b) or for protein with Coomassie Blue. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed according to Laemmli (1970), and protein was stained with Coomassie Blue. All chemicals were reagent grade, and water was deionized and distilled.

Liver specimens were obtained at autopsy within 12 h of death from adult Japanese individuals who had no history of liver disease. The majority died from heart disease. The liver specimens were collected in Chiba City and Tokyo, Japan, and Honolulu, HI. All donors from Hawaii had documented Japanese ancestry. The specimens were kept frozen and delivered by air freight to Indianapolis, IN. They were stored at −55 °C until use.

Alcohol dehydrogenase isoenzymes were isolated by affinity and ion-exchange chromatography (Lange & Vallee, 1976; Bosron et al., 1983b). Briefly, livers were homogenized in two volumes of distilled water and homogenates were centrifuged at 69000*g* for 60 min. The homogenate–supernatant was passed through a column containing 5 mL of packed DEAE-cellulose/g of liver (DE-52; Whatman, Clifton, NJ), equilibrated with 10 mM Tris-HCl at pH 8.0. Tris-HCl buffer, pH 9.0, and NAD^+ were added to the effluent to a concentration of 100 mM and 3 mM, respectively, and solution was applied to a CapGapp–Sepharose affinity column (Lange & Vallee, 1976; Yoshida et al., 1981; Yin et al., 1984). Isoenzymes composed of α, β, and γ subunits were eluted with 0.5 M ethanol in 100 mM Tris-HCl, pH 7.5. The recovery of activity in this purification step was 80–95%. Separation of isoenzymes was accomplished by ion-exchange chromatography on 30 × 1.5 cm CM-cellulose (CM-52; Whatman, Clifton, NJ) in 5 mM Tris-HCl, 1.0 mM NAD^+, and 1.0 mM dithiothreitol, pH 8.7 at 4 °C, with a linear gradient of 0–31 mM NaCl in buffer. The recovery of activity ranged from 75 to 85%. Mixtures of isoenzymes were further purified by chromatography on CM-cellulose equilibrated in 15 mM NaP_i, 10 mM ethanol, and 1 mM dithiothreitol, pH 6.7 at 4 °C, with a 15–35 mM linear gradient of buffer. The recovery of activity ranged from 65 to 75%. Separation of the mixture of $\beta_2\beta_2$, $\beta_2\beta_1$, and $\beta_1\beta_1$ was accomplished by affinity chromatography on agarose–hexane–AMP (type 2; P-L Biochemicals, Milwaukee, WI) in 100 mM Tris-HCl and 1 mM dithiothreitol, pH 8.8 at 4 °C, with a linear gradient of 0–100 μM NADH in buffer, followed by 100 mM NaCl and 100 μM NADH in buffer. The recovery of activity ranged from 80 to 90%. Fractions containing single isoenzymes were precipitated with $(NH_4)_2SO_4$, 0.6 g/mL, centrifuged, and stored at −55 °C. All molecular forms were gel filtered on Bio-Gel P-6 (Bio-Rad, Richmond, CA) in 10 mM NaP_i, pH 7.5, before further experiments were performed.

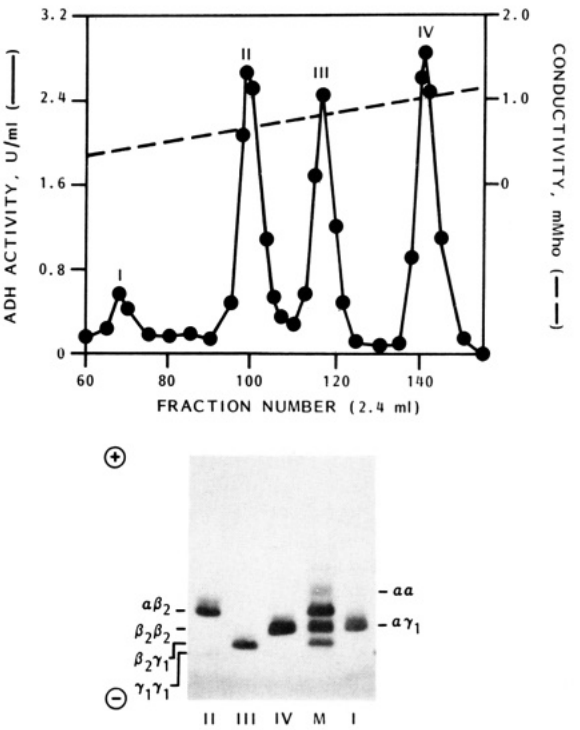

FIGURE 1: Separation and isoelectric focusing of ADH isoenzymes from a liver with the ADH_2 2-2, ADH_3 1-1 phenotype. The top panel shows the activity elution profile for the separation of these isoenzymes by chromatography on CM-cellulose at pH 8.7. The lower panel shows the agarose isoelectric focusing patterns of the four activity peaks: lane I, fraction 68; lane II, fraction 99; lane III, fraction 117; lane IV, fraction 141; lane M, the mixture of isoenzymes applied to CM-cellulose. The isoenzymes were stained for ethanol oxidizing activity.

Purified isoenzymes were dissociated into subunits by freezing in 0.1 M NaP_i, 1.0 M NaCl, 0.4 M sucrose, and 0.1 M 2-mercaptoethanol, pH 7.0, for 12 h at −55 °C and reassociated by rapid thawing at 25 °C and dialyzing the sample for 24 h at 4 °C against two changes of 10 mM NaP_i, 1 mM dithiothreitol, and 50 μM $ZnSO_4$, pH 7.0 (Bosron et al., 1983b). Recovery of activity ranged from 30 to 60%. The patterns of dissociated–recombined isoenzymes were examined by starch gel electrophoresis and isoelectric focusing.

Steady-state kinetic studies were performed in 0.1 M NaP_i, pH 7.5 or 6.0 at 25 °C, with a Cary 210 spectrophotometer at 0.02–0.05 full-scale absorbency. All kinetic data were obtained in duplicate or triplicate, and steady-state kinetic constants were evaluated with the statistical programs of Cleland (1979). The coefficients of variation for K_m, K_i, and V_{max} were 15% or less. The concentration of enzyme active sites were determined by fluorescence titration of the binary enzyme–isobutyramide complex with NADH (Yonetani & Theorell, 1962). V_{max} values are expressed as per minute (min^{-1}); i.e., micromoles of NADH produced or utilized per minute per micromole of enzyme active site.

Results

Isolation and Dissociation–Recombination of β_2-Containing Alcohol Dehydrogenase Isoenzymes from a Liver with the Homozygous ADH_2 2-2 Phenotype. The agarose isoelectric focusing pattern of the isoenzymes present in a Japanese liver with the homozygous ADH_2 2-2 phenotype is shown in Figure 1, lane M. The mixture of isoenzymes were purified by affinity chromatography on CapGapp–Sepharose (Lange & Vallee, 1976; Yin et al., 1984) and subsequently separated into four

[2] Abbreviations: P_i, inorganic orthophosphate; PP_i, inorganic pyrophosphate; Tris, tris(hydroxymethyl)aminomethane; CapGapp–Sepharose, 4-[3-[(6-aminocaproyl)amino]propyl]pyrazole–Sepharose; DEAE-cellulose, *O*-(diethylaminoethyl)cellulose; CM-cellulose, *O*-(carboxymethyl)cellulose.

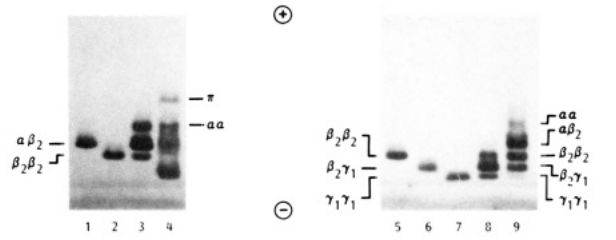

FIGURE 2: Agarose isoelectric focusing of $\alpha\beta_2$ and $\beta_2\gamma_1$ before and after dissociation–recombination treatment. The isoelectric focusing patterns of $\alpha\beta_2$ and $\beta_2\gamma_1$ are shown in lanes 1 and 6, respectively, and their patterns after dissociation–recombination treatment are shown in lanes 3 and 8, respectively. Patterns of homogenate–supernatants from a liver with the ADH_2 1-1, ADH_3 1-1 phenotype which has high π and $\alpha\alpha$ enzyme activity and of isoenzymes bound to CapGapp–Sepharose from a liver with the ADH_2 2-2, ADH_3 1-1 phenotype are shown in lanes 4 and 9, respectively. Purified $\beta_2\beta_2$ is shown in lanes 2 and 5 and purified $\gamma_1\gamma_1$ in lane 7. The isoenzymes were stained for ethanol oxidizing activity.

peaks of enzyme activity by ion-exchange chromatography on CM-cellulose at pH 8.7 (Figure 1). Under these conditions, the $\beta\beta$ isoenzymes elute last from the ion-exchange column (Yoshida et al., 1981; Yin et al., 1984). Enzyme recovered under peak IV (Figure 1) exhibited a single band on agarose isoelectric focusing (Figure 1, lane IV), and it had an atypical pH 10/8.5 activity ratio of 0.30. After dissociation-recombination treatment, this isoenzyme remained as a single band on focusing, suggesting that it was $\beta_2\beta_2$. Peak I exhibited a single activity band on isoelectric focusing (Figure 1, lane I), and it had a typical pH 10/8.5 activity ratio of 1.57. Its mobility on starch gel electrophoresis was identical with that of $\alpha\gamma_1$ (Bosron et al., 1983b). Enzyme under peak II (Figure 1) exhibited an atypical pH–activity ratio of 0.52. After rechromatography of peak II on CM-cellulose at pH 6.7 (elution profile not shown), a minor component with a typical pH–activity ratio, 1.50, was separated from the major atypical component. The minor component was identified as $\gamma_1\gamma_1$ by starch gel electrophoresis, and it exhibited the greatest cathodic mobility on agarose isoelectric focusing of all isoenzymes present in this liver. The major component under peak II was the heterodimer $\alpha\beta_2$, since it generated $\alpha\alpha$ and $\beta_2\beta_2$ after dissociation–recombination (Figure 2, lanes 1 and 3). Peak III contained a single isoenzyme (Figure 1, lane III) with an atypical pH–activity ratio of 0.62. This isoenzyme was $\beta_2\gamma_1$, since $\beta_2\beta_2$ and $\gamma_1\gamma_1$ were formed after dissociation–recombination of the heterodimer (Figure 2, lanes 6 and 8).

The conditions used in this study for the separation of isoenzymes from livers with the ADH_2 2-2, ADH_3 1-1 phenotype were modified from those reported previously (Yin et al., 1984). The inclusion of 1 mM dithiothreitol in the CM-cellulose elution buffers (Figure 1) resulted in better separation of $\alpha\beta_2$ and $\beta_2\gamma_1$; however, the separation of $\gamma_1\gamma_1$ from $\alpha\beta_2$ was better when the chromatography was performed in buffers without dithiothreitol (Yin et al., 1984). Each of the five purified isoenzymes exhibited a single protein band of approximately 40 000 daltons on sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

A sixth isoenzyme with mobility corresponding to $\alpha\alpha$ was detected in the homogenate–supernatant of this liver by isoelectric focusing (Figure 1, lane M); however, $\alpha\alpha$ was not recovered after CM-cellulose chromatography. In other preparations from livers with the same phenotype, small amounts of $\alpha\alpha$ eluted from CM-cellulose before peak I and immediately after application of the NaCl gradient.

Isolation and Dissociation–Recombination of β_2-Containing Alcohol Dehydrogenase Isoenzymes from a Liver with the

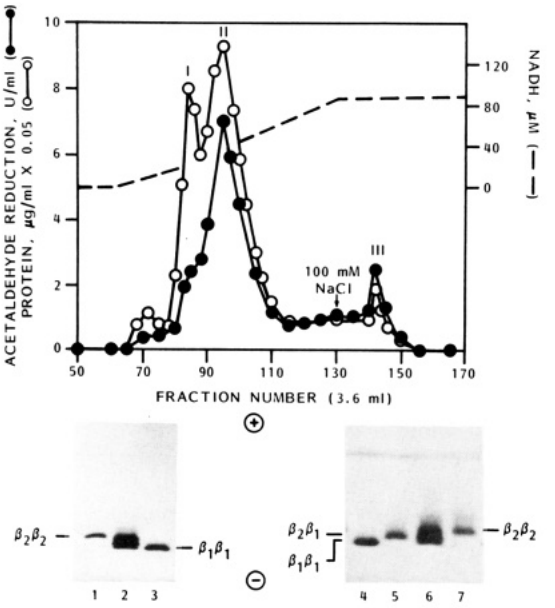

FIGURE 3: Separation and isoelectric focusing of $\beta_2\beta_2$, $\beta_2\beta_1$, and $\beta_1\beta_1$. The $\beta\beta$ mixture was recovered under the last peak after CM-cellulose chromatography at pH 8.7 of a heterozygous ADH_2 2-1 phenotype liver. The elution profiles of acetaldehyde reducing activity measured at pH 6.0 and protein after chromatography of the $\beta\beta$ mixture on agarose–hexane–AMP at pH 8.8 are shown in the upper panel. The isoelectric focusing patterns of $\beta_1\beta_1$ in agarose–hexane–AMP fraction 81, $\beta_2\beta_1$ in fractions 98–110, and $\beta_2\beta_2$ in fractions 140–150 are shown in lanes 4, 5, and 7, respectively. For comparison, $\beta_2\beta_2$, the $\beta\beta$ mixture, and $\beta_1\beta_1$ are shown in lane 1, lanes 2 and 6, and lane 3, respectively. The isoenzymes were stained for protein by Coomassie Blue.

Heterozygous ADH_2 2-1 Phenotype. The CM-cellulose elution profile of isoenzymes from a liver with the heterozygous ADH_2 2-1 phenotype was similar to that of isoenzymes from the homozygous liver shown in Figure 1, except that $\alpha\beta_1$ coeluted with $\alpha\beta_2$ in peak II, $\beta_1\gamma_1$ coeluted with $\beta_2\gamma_1$ in peak III, and two other isoenzymes eluted with $\beta_2\beta_2$ in peak IV (lanes 2 and 6, Figure 3). The most anodic isoenzyme in peak IV had a mobility on isoelectric focusing identical with that of $\beta_2\beta_2$ (lanes 1 and 7, Figure 3), the most cathodic isoenzyme had a mobility identical with that of $\beta_1\beta_1$ (lanes 3 and 4, Figure 3), and the third isoenzyme, exhibiting an intermediate mobility (lane 5, Figure 3), was presumed to be the heterodimer $\beta_2\beta_1$ (Yin et al., 1984).

To verify this assignment, we attempted to separate the three $\beta\beta$ isoenzymes by chromatography on ion-exchange, chromatofocusing, and affinity resins. They could not be separated by CM-cellulose chromatography or by chromatofocusing; however, they were partially resolved by elution from the affinity resin agarose–hexane–AMP with a gradient of 0–100 μM NADH followed by application of 0.1 M NaCl in 100 μM NADH (Figure 3). As shown in lanes 4 and 7 of Figures 3, isoenzymes $\beta_1\beta_1$ and $\beta_2\beta_2$ were isolated under agarose–hexane–AMP peaks I and III, respectively. The isoenzyme under peak II was $\beta_2\beta_1$, since $\beta_2\beta_2$ and $\beta_1\beta_1$ were generated after dissociation–recombination (Figure 4, lanes 4 and 1). The heterodimer $\beta_2\beta_1$ could be produced in vitro by dissociation–recombination of purified $\beta_2\beta_2$ plus $\beta_1\beta_1$ as shown in lanes 5–7 of Figure 4.

Kinetic Properties of $\beta_2\beta_2$, $\beta_2\beta_1$, $\alpha\beta_2$, and $\beta_2\gamma_1$. The pH–activity profiles of $\beta_2\beta_2$, $\beta_2\beta_1$, $\alpha\beta_2$, and $\beta_2\gamma_1$ for ethanol oxidation all exhibited a single optimum at 8.5–8.8 which is similar to that of the isoenzyme mixture in atypical liver homogenate–supernatants (Berger et al., 1974; Bosron et al., 1980). The specific activities of the four β_2-containing isoenzymes for ethanol oxidation at pH 8.5 are shown in Table

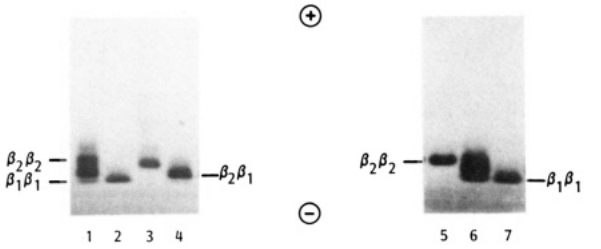

FIGURE 4: Agarose isoelectric focusing of $\beta_2\beta_1$ and a mixture of $\beta_2\beta_2$ plus $\beta_1\beta_1$ after dissociation–recombination treatment. The isoelectric focusing patterns of $\beta_2\beta_1$ and a mixture of $\beta_2\beta_2$ plus $\beta_1\beta_1$ (protein ratio of $\beta_2\beta_2$:$\beta_1\beta_1$ = 1:9) after dissociation–recombination are shown in lanes 1 and 6, respectively. For comparison purified $\beta_1\beta_1$ is shown in lanes 2 and 7, $\beta_2\beta_2$ in lanes 3 and 5, and $\beta_2\beta_1$ in lane 4. The isoenzymes were stained for ethanol oxidizing activity.

Table I: Comparison of Specific Activities of Homo- and Heterodimeric Isoenzymes for Ethanol Oxidation at pH 8.5[a]

		observed activity (units/mg)	calculated activity[b] (units/mg)
homodimer	$\beta_2\beta_2$	19	
	$\beta_1\beta_1$	0.09	
	$\alpha\alpha$	1.9	
	$\gamma_1\gamma_1$	2.1	
heterodimer	$\beta_2\beta_1$	9.3	9.6
	$\alpha\beta_2$	12	11
	$\beta_2\gamma_1$	10	11

[a] Enzyme activity was determined with 33 mM ethanol and 2.4 mM NAD^+ in 33 mM glycine–NaOH, pH 8.5 at 25 °C. Protein concentration was determined with the Folin reagent. Values of $\alpha\alpha$ and $\gamma_1\gamma_1$ are from Bosron et al. (1980). [b] Heterodimer values are the mean of the respective homodimer values, i.e., $v_{\beta_2\beta_1} = (v_{\beta_2\beta_2} + v_{\beta_1\beta_1})/2$.

Table II: Kinetic Constants for Homodimeric Alcohol Dehydrogenase Isoenzymes at pH 7.5[a]

constant	$\beta_2\beta_2$	$\beta_1\beta_1$	$\alpha\alpha$	$\gamma_1\gamma_1$	$\gamma_2\gamma_2$
K_m(ethanol) (mM)	0.94[b]	0.049	4.2	1.0	0.63
K_m(acetaldehyde) (mM)	0.24[c]	0.085	4.3	0.33	0.24
K_m(NAD^+) (μM)	180[d]	7.4	13	7.9	8.7
K_i(NAD^+) (μM)	340[e]	90	32		
K_m(NADH) (μM)	105[f]	6.4	11	7.0	
K_i(NADH) (μM)	9.7[g]	0.19	0.40	0.98	1.6
$V_{max,forward}$ (min^{-1})	400[b]	9.2	27	87	35
$V_{max,reverse}$ (min^{-1})	3900[c]	240	700	2100	1400

[a] Enzyme activity was determined in 0.1 M NaP_i, pH 7.5 at 25 °C. Values for $\beta_2\beta_2$ are the mean for two isoenzyme preparations and values for $\beta_1\beta_1$, $\alpha\alpha$, $\gamma_1\gamma_1$, and $\gamma_2\gamma_2$ are from Bosron et al. (1983b). [b] NAD^+ concentration was 2.4 mM. K_m and V_{max} values were calculated from the SUBIN program of Cleland (1979). [c] NADH was 0.2 mM. K_m and V_{max} values were calculated from SUBIN. [d] Ethanol was 20 mM, and K_m was calculated from HYPER. [e] Ethanol and NAD^+ were covaried, and K_i was calculated from SEQUEN. [f] Acetaldehyde was 4 mM, and K_m was calculated from HYPER. [g] Ethanol was 20 mM, and K_i was calculated from COMP.

I. The pH–activity optima for acetaldehyde reduction of $\beta_2\beta_2$ and $\beta_1\beta_1$ were at 7.4 and 5.9, respectively, while the heterodimer $\beta_2\beta_1$ had an optimum at 7.4 and a distinct shoulder at about 5.9 (Figure 5).

The steady-state kinetic constants of $\beta_2\beta_2$ for substrates and coenzymes were determined at pH 7.5 for two different isoenzyme preparations (Table II). The substrate saturation curves of $\beta_2\beta_2$ for ethanol oxidation and acetaldehyde reduction did not fit simple Michaelis–Menten kinetics but exhibited substrate inhibition; consequently, K_m, K_i, and V_{max} values were calculated from the fit of data to a second-order polynomial expression for substrate inhibition (Cleland, 1979; Bosron et al., 1983b). K_m and V_{max} values are shown in Table II. The substrate inhibition constants for ethanol and acetaldehyde were 340 and 205 mM, respectively. Linear intersecting

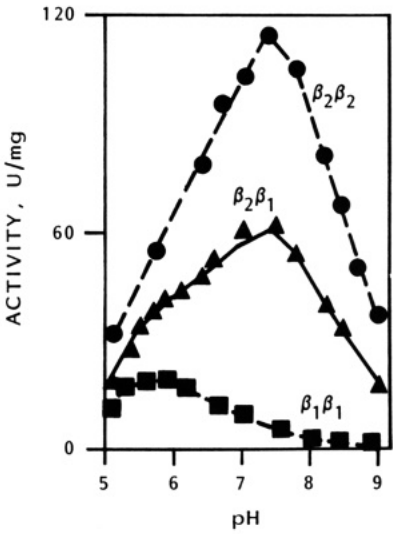

FIGURE 5: pH–activity profiles for acetaldehyde reduction of $\beta_2\beta_2$, $\beta_2\beta_1$, and $\beta_1\beta_1$. Acetaldehyde reducing activity was determined with 5 mM acetaldehyde and 0.3 mM NADH in 33 mM NaP_i, pH 5–8, and 33 mM glycine adjusted to pH 8–9 with NaOH at 25 °C. The solid line represents half the activities of $\beta_2\beta_2$ plus $\beta_1\beta_1$ at indicated pH values.

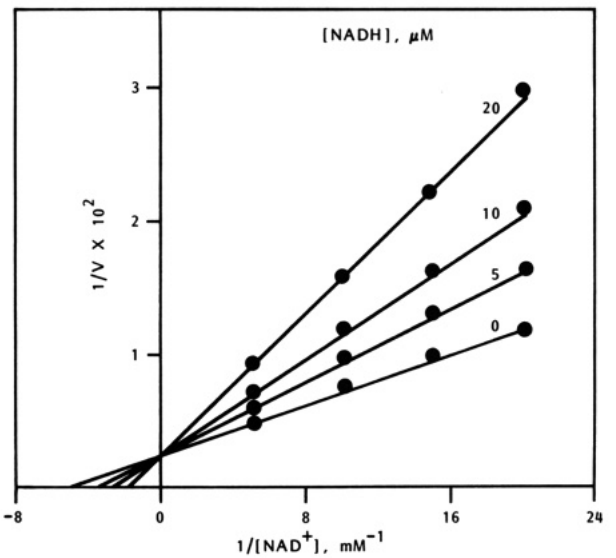

FIGURE 6: Product inhibition of $\beta_2\beta_2$ by NADH. Enzyme activity was determined in 0.1 M NaP_i, pH 7.5 at 25 °C, with 20 mM ethanol. Activity, V, is expressed as min^{-1}. The solid lines are the fit of data of the COMP program of Cleland (1979).

double-reciprocal plots were obtained when NAD^+ and ethanol concentrations were covaried (ethanol < 5 mM). NADH was a competitive inhibitor of NAD^+ at 20 mM ethanol (Figure 6), and ethanol was a mixed-noncompetitive inhibitor of acetaldehyde at 0.2 mM NADH.

At pH 6.0, both $\beta_2\beta_2$ and $\beta_1\beta_1$ obeyed simple Michaelis–Menten kinetics for NADH reduction with 4 mM acetaldehyde. The K_m values of $\beta_2\beta_2$ and $\beta_1\beta_1$ for NADH were 40 and 5.2 μM, respectively, and the V_{max} values were 2300 and 410 min^{-1}, respectively (Table III). The velocities of $\beta_2\beta_1$ were intermediate between $\beta_2\beta_2$ and $\beta_1\beta_1$ over a wide range of NADH concentration, 3.4–190 μM (Figure 7), and reciprocal plots (not shown) indicated that the kinetics of heterodimer $\beta_2\beta_1$ were nonlinear. Apparent kinetic constants for the β_1 and β_2 subunits in the heterodimer were estimated from the fit of data at low NADH concentration, 3.4–15 μM, and high NADH concentration, 29–190 μM (Cleland, 1979). As shown in Table III, initial estimates of K_m and V_{max} of β_1 were 16

Table III: Estimation of the NADH Kinetic Constants of β_2 and β_1 Subunits from Kinetics of $\beta_2\beta_1$, $\beta_2\beta_2$, and $\beta_1\beta_1$ at pH 6.0

	β_2 subunit		β_1 subunit	
source of estimate	K_m (μM)	V_{max} (min^{-1})	K_m (μM)	V_{max} (min^{-1})
kinetics of $\beta_2\beta_1$, 29–190 μM NADH	30 ± 3	1400 ± 40		
kinetics of $\beta_2\beta_1$, 3.4–15 μM NADH			16 ± 3	1200 ± 100
correction of subunit kinetics at high and low NADH concentration	43 ± 5	1200 ± 50	6.0 ± 2.0	290 ± 40
kinetics of $\beta_2\beta_2$, 3.1–190 μM NADH	40 ± 1	1150 ± 10[a]		
kinetics of $\beta_1\beta_1$, 3.1–190 μM NADH			5.2 ± 0.3	205 ± 5[a]

[a] V_{max} values for subunits are half the values for homodimers.

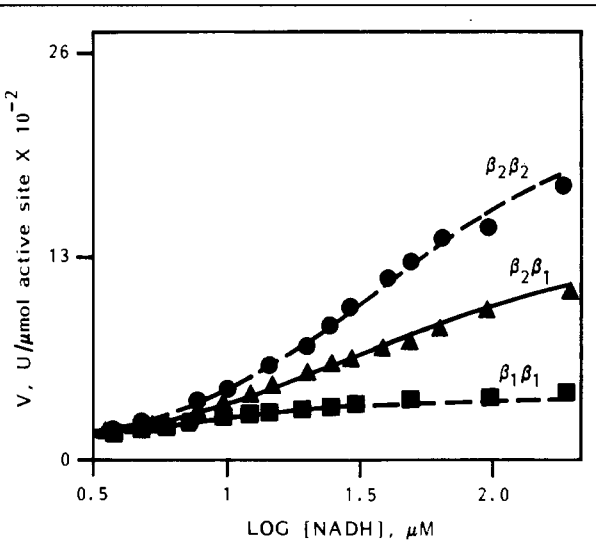

FIGURE 7: Dependency of acetaldehyde reducing activity of $\beta_2\beta_2$, $\beta_2\beta_1$, and $\beta_1\beta_1$ on NADH concentration. Enzyme activity was determined in 0.1 M NaP_i, pH 6.0 at 25 °C, with 4 mM acetaldehyde. The solid line is the fit of data to $v_{\beta_2\beta_1} = (1/2)\ [(V_{max,\beta_1\beta_1}[S])/(K_{m,\beta_1\beta_1} + [S]) + (V_{max,\beta_2\beta_2}[S])/(K_{m,\beta_2\beta_2} + [S])]$ by utilizing the K_m and $V_{max,reverse}$ values of $\beta_2\beta_2$ and $\beta_1\beta_1$ shown in Table III.

μM and 1200 min^{-1}, and for β_2 they were 30 μM and 1400 min^{-1}, respectively. Since the contribution of β_2 to β_1 activity at the low NADH concentrations is large, the activity of β_2 was calculated at 3.4–15 μM NADH by using the initial estimates of its kinetic constants, and these values were subtracted from the observed velocities. The kinetic constants for the low K_m β_1 subunit were then recalculated by utilizing these corrected velocities as described by the procedure of Spears et al. (1971). Similarly, the contribution of β_1 to β_2 activity at high NADH concentration was calculated from the initial estimates of its kinetic constants, and these values were subtracted from the observed velocities at 29–190 μM NADH. Kinetic constants for the high K_m β_2 subunit were then recalculated from the corrected velocities. After the calculations were repeated 3 times, the corrected K_m and V_{max} values for β_2 were 43 μM and 1200 min^{-1} and values for β_1 were 6.0 μM and 290 min^{-1}, respectively (Table III). Because large corrections were made to the activities of β_1 at 3.4–15 μM NADH, the standard deviations for recalculated kinetic constants were large. Nonetheless, the K_m and V_{max} values estimated for the β_2 and β_1 subunits in $\beta_2\beta_1$ by this method agreed well with values obtained for the subunits in the $\beta_2\beta_2$ and $\beta_1\beta_1$ homodimers (Table III). As shown by the solid line in Figure 7, the velocities of $\beta_2\beta_1$ calculated from the sum of Michaelis–Menten expressions for β_2 and β_1 in homodimers also agreed well with the observed velocities for $\beta_2\beta_1$.

Discussion

von Wartburg et al. (1965) first reported the characterization of a partially purified preparation of atypical human liver alcohol dehydrogenase that exhibited a pH optimum for ethanol oxidation at 8.5–8.8. A variant isoenzyme migrating cathodic to $\beta_1\beta_1$ on starch gel electrophoresis was separated from the other forms by elution from a CM-cellulose column with high salt at pH 9.0 (Schenker et al., 1971). It was proposed that this cathodic isoenzyme corresponds to the homodimer $\beta_2\beta_2$ that is produced by ADH_2^2 (Smith et al., 1971). Subsequent structural studies revealed that the cathodic isoenzyme isolated by Schenker et al. (1971) was actually an unresolved mixture of $\beta_2\beta_2$ and $\beta_2\beta_1$ obtained from a liver with the heterozygous ADH_2 2-1 phenotype (Berger et al., 1974). It then became evident that new methodology for the identification of livers exhibiting the homozygous and heterozygous ADH_2 phenotypes and the separation of atypical isoenzymes must be developed before the kinetic and structural properties of pure $\beta_2\beta_2$ can be investigated.

We have recently shown that Japanese livers containing β_2 isoenzymes can be clearly differentiated into two atypical phenotypes, the homozygous ADH_2 2-2 and the heterozygous ADH_2 2-1 phenotypes, by isoelectric focusing on agarose gels (Yin et al., 1984). With the ADH_2 2-2 livers, the last activity peak eluting from CM-cellulose with high salt at pH 8.7 (Figure 1, fraction IV) exhibited a single band on isoelectric focusing; it was $\beta_2\beta_2$. The last activity peak with the ADH_2 2-1 livers exhibited three bands on agarose isoelectric focusing (Figure 3); they were $\beta_1\beta_1$, $\beta_2\beta_2$, and a band with a mobility intermediate between the homodimers which we presumed to be $\beta_2\beta_1$ (Yin et al., 1984). In agreement with the observations of Berger et al. (1974), the mixture of isoenzymes with β_2 and β_1 subunits from the heterozygous liver could not be separated by chromatography on CM-cellulose at pH values ranging from 6.5 to 9.2; however, the isoenzymes could be separated by affinity chromatography on agarose–hexane–AMP (Figure 3). An isoenzyme with an atypical pH optimum for activity was separated from $\beta_1\beta_1$ by elution from the column with a gradient of increasing NADH concentration (peak II, Figure 3); it was found to be $\beta_2\beta_1$ by dissociation–recombination experiments (Figure 4). The homodimer $\beta_2\beta_2$ eluted from agarose–hexane–AMP only with the application of a high concentration of NaCl (peak III, Figure 3). These data indicate that CM-cellulose is effective for the isolation of $\beta_2\beta_2$ only from livers with the homozygous ADH_2 2-2 phenotype, and other methods such as agarose–hexane–AMP chromatography are required for livers with the heterozygous ADH_2 2-1 phenotype. Therefore, previous studies of enzyme presumed to be $\beta_2\beta_2$ that had employed CM-cellulose chromatography for the separation of isoenzymes from livers not positively identified as the ADH_2 2-2 phenotype may be questionable (Yoshida et al., 1981; Schenker et al., 1971; Okuda & Okuda, 1983).

All the isoenzymes present in the Japanese livers with the ADH_2 2-2, ADH_3 1-1 phenotype were purified to homogeneity and examined by agarose isoelectric focusing and starch gel electrophoresis (Figures 1 and 2; Yin et al., 1984). We have followed the scheme of Smith et al. (1971) for naming these isoenzymes. As verified by dissociation–recombination experiments (Figure 2), the homozygous ADH_2 2-2, ADH_3, 1-1

livers contain three isoenzymes with pH optima at 8.5–8.8, $\alpha\beta_2$, $\beta_2\gamma_1$, and $\beta_2\beta_2$, that are produced by random combination of α, β_2, and γ_1 subunits. Livers with the heterozygous ADH_2 2-1, ADH_3 1-1 phenotype contain $\alpha\beta_2$, $\beta_2\gamma_1$, $\beta_2\beta_2$, and a fourth isoenzyme with a pH optimum for ethanol oxidation at 8.5–8.8, $\beta_2\beta_1$.

The reaction mechanism of purified $\beta_2\beta_2$ was determined by steady-state kinetic studies at pH 7.5. The converging reciprocal pattern when both NAD^+ and ethanol were covaried, the competitive inhibition of NAD^+ reduction by NADH (Figure 6), and the mixed noncompetitive inhibition of ethanol oxidation by acetaldehyde at saturating NAD^+ concentration are consistent with the ordered sequential mechanism with coenzyme adding first as proposed for horse liver alcohol dehydrogenase (Wratten & Cleland, 1963; Dworschack & Plapp, 1977) and other human liver alcohol dehydrogenase isoenzymes (Dubied et al., 1977; Bosron et al., 1979a, 1983b). The rate constant for NADH dissociation, k_{-4}, was calculated to be 360 min^{-1} from the kinetic constants in Table II and the equation (Cleland, 1963)

$$k_{-4} = V_{max,reverse}K_i(NADH)/K_m(NADH)$$

Since k_{-4} is nearly equal to V_{max} for ethanol oxidation, 400 min^{-1} (Table II), NADH dissociation appears to be rate limiting for $\beta_2\beta_2$ as has been demonstrated for horse liver alcohol dehydrogenase (Dalziel & Dickerson, 1966; Wratten & Cleland, 1963; Dworschack & Plapp, 1977) and the human liver $\alpha\alpha$ and $\beta_1\beta_1$ isoenzymes (Bosron et al., 1983b).

The purified homodimer $\beta_2\beta_2$ has a pH optimum for ethanol oxidation at 8.5–8.8. Even though the pH–activity optima for $\beta_1\beta_1$, $\alpha\alpha$, and $\gamma_1\gamma_1$ are at 10.0–10.5 (Bosron et al., 1980, 1983b), the pH–activity profiles for the three β_2-containing heterodimers $\beta_2\beta_1$, $\alpha\beta_2$, and $\beta_2\gamma_1$ are similar to that of $\beta_2\beta_2$. This is because the specific activities of $\alpha\alpha$, $\beta_1\beta_1$, and $\gamma_1\gamma_1$ are lower than that of $\beta_2\beta_2$ especially at pH 8.5 (Table I). Hence, the contribution of the α, β_1, and γ_1 subunits to the total activity of the three β_2-containing heterodimers at pH 8.5 is small.

The activity profile for acetaldehyde reduction by $\beta_2\beta_1$ had an optimum at pH 7.4 and a distinct shoulder at pH 5.9 (Figure 5). As shown by the solid line in Figure 5, activity calculated from half the activities of $\beta_2\beta_2$ and $\beta_1\beta_1$ at pH values from 5.0 to 9.0 agreed well with the data for purified $\beta_2\beta_1$. Both $\beta_2\beta_2$ and $\beta_1\beta_1$ obeyed simple Michaelis–Menten kinetics when NADH concentration was varied at pH 6.0, but double-reciprocal plots of $\beta_2\beta_1$ were nonlinear. The extrapolated K_m values for the β_2 and β_1 subunits in the heterodimer, 43 and 6.0 μM, respectively, agreed well with values for the subunits in the $\beta_2\beta_2$ and $\beta_1\beta_1$ homodimers, 40 and 5.2 μM, respectively (Table III). Moreover, velocities of $\beta_2\beta_1$ agreed well with those calculated from a two-term Michaelis–Menten equation utilizing the K_m and V_{max} values determined for subunits in the two homodimers (Figure 7). The data suggest that enzyme activity can be predicted on the basis of the assumption that the subunits in $\beta_2\beta_1$ act in an independent, noncooperative fashion. However, some of the kinetic properties of other alcohol dehydrogenase heterodimers formed by the combination of β_1, γ_1, or γ_2 subunits apparently cannot be predicted from properties of these subunits in homodimers (Wagner et al., 1983) perhaps because $\gamma_1\gamma_1$ and $\gamma_2\gamma_2$ exhibit negative cooperativity for ethanol saturation (Bosron et al., 1983b).

The kinetic properties of $\beta_2\beta_2$ at pH 7.5 (Table II) are substantially different from those of four other homodimeric class I isoenzymes, $\alpha\alpha$, $\beta_1\beta_1$, $\gamma_1\gamma_1$, and $\gamma_2\gamma_2$ (Bosron et al., 1983b). At a near-physiological pH of 7.5 and optimal concentrations of ethanol and NAD^+, the V_{max} of $\beta_2\beta_2$ is 5–40 times greater than that of the four other homodimers. It has been reported that Japanese and Chinese have higher average ethanol elimination rates than whites (Reed et al., 1976; Hanna, 1978). These observations are consistent with the 85% frequency of the high-activity β_2-containing isoenzymes in Japanese and Chinese (Stamatoyannopoulos et al., 1975; Teng et al., 1979; Harada et al., 1980; Yin et al., 1984) as compared to their low frequency in whites (Smith et al., 1971; Harada et al., 1978; Bosron & Li, 1981).

As shown in Table II, K_m and K_i values of $\beta_2\beta_2$ for NAD^+ and NADH are significantly greater than the corresponding values of $\alpha\alpha$, $\beta_1\beta_1$, $\gamma_1\gamma_1$, and $\gamma_2\gamma_2$. Jörnvall et al. (1984) recently reported that the active site arginine-47 in $\beta_1\beta_1$ and $\gamma_1\gamma_1$ is replaced by histidine in $\beta_2\beta_2$. The sequence of $\alpha\alpha$ and $\gamma_2\gamma_2$ at this site has not been determined. The substitution of a weaker base, histidine, in the NAD(H) anion binding site of $\beta_2\beta_2$ would be consistent with the higher K_i (i.e., the dissociation constant for binary enzyme–coenzyme complexes) and K_m values of $\beta_2\beta_2$ for NAD^+ and NADH as compared with the values for $\beta_1\beta_1$ and $\gamma_1\gamma_1$ (Table II). Since NADH dissociation is the rate-limiting step for ethanol oxidation by $\beta_2\beta_2$ and $\beta_1\beta_1$, the higher V_{max} value for $\beta_2\beta_2$ is also consistent with the histidine for arginine substitution in the active site.

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Registry No. NAD^+, 53-84-9; NADH, 58-68-4; acetaldehyde, 75-07-0; ethanol, 64-17-5; alcohol dehydrogenase, 9031-72-5.

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Does Pyridoxal 5′-Phosphate Function in Glycogen Phosphorylase as an Electrophilic or a General Acid Catalyst?†

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ABSTRACT: α-D-Glucose 1-diphosphate interacts with pyridoxal-reconstituted rabbit muscle phosphorylase *b* activated by AMP (AMP-S). Under these conditions, the glucose moiety of α-D-[^{14}C]glucose 1-diphosphate is transferred to limit dextrin forming α(1→4) glycosidic bonds and simultaneously releasing pyrophosphate as shown by ^{31}P NMR spectroscopy. Thus, specific structural requirements invoked to explain the reactions of pyridoxal(5′)diphospho(1)-α-D-glucose need not to be assumed in the case of the reactions of α-D-glucose 1-diphosphate. Dianions isomorphous to phosphate activate pyridoxal phosphorylase regardless of their p*K* values while the same anions, when bound covalently to pyridoxal, are inactive. Thus, anions bound noncovalently to pyridoxal phosphorylase act differently than anions linked covalently to pyridoxal, such as the 5′-phosphate group of pyridoxal 5′-phosphate, which is postulated to be part of a proton donor–acceptor pathway. The reaction of 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol (heptenitol) with phosphorylase yields, in the presence of orthophosphate as a glycosyl acceptor, 1-deoxy-D-*gluco*-heptulose 2-phosphate (heptulose-2-P). This sugar phosphate is unreactive but a potent competitive inhibitor for rabbit muscle phosphorylase *b* and potato phosphorylase with respect to α-D-glucose 1-phosphate: $K_i = 14 \times 10^{-6}$ M and 1.9×10^{-6} M, respectively. Heptulose-2-P is ideally suited for ^{31}P NMR experiments with phosphorylase because its phosphate resonance and that of the 5′-phosphate of pyridoxal 5′-phosphate do not overlap, and in contrast to α-D-glucopyranose cyclic 1,2-phosphate, another powerful inhibitor of glycogen phosphorylases, heptulose-2-P is protonatable in the pH range of the enzymatic reaction. Use was made of this property of heptulose-2-P in ^{31}P NMR investigations, the results of which indicated that the dianionic 5′-phosphate group of the natural cofactor becomes partially protonated on binding of heptulose-2-P to potato phosphorylase, whereas the monoprotonated 5′-phosphonate group of the partially active 5′-deoxypyridoxal-5′-(methylenephosphonate) muscle phosphorylase *b* derivative was shown to share a proton with the phosphate moiety of heptulose-2-P. While the experiments support a role for the cofactor phosphate in glycogen phosphorylases in a general acid–base catalysis [cf. Feldmann, K., Hörl, M., Klein, H. W., & Helmreich, E. J. M. (1978) *Proc. FEBS Meet. 42*, 205–218], they do not uphold a function of the phosphorus of the 5′-phosphate group of the cofactor as an electrophilic catalyst [cf. Withers, S. G., Madsen, N. B., Sykes, B. D., Takagi, M., Shimomura, S., & Fukui, T. (1981) *J. Biol. Chem. 256*, 10759–10762].

Our preceding studies of phosphorylase reactions with glycosylic[1] substrates such as D-glucal, heptenitol, and glucosyl fluoride indicated that α-glucan phosphorylases are general acid–base catalysts requiring, in addition to suitably charged amino acid side chains, phosphate (arsenate) and a 5′-phosphate anion linked covalently to pyridoxal (Klein et al., 1982, 1984; Palm et al., 1983). On the basis of these experiments, a role for the 5′-phosphate group of the cofactor as a general acid was proposed. In disagreement with this hypothesis, Madsen and colleagues (Withers et al., 1981b, 1982a; Takagi

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[1] According to Hehre et al. (1980), glycosylic substrates are compounds that yield a glycosyl residue on protonation.

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