Kinetic Studies on Benzyl Alcohol Dehydrogenase Encoded by TOL Plasmid pWW0

A MEMBER OF THE ZINC-CONTAINING LONG CHAIN ALCOHOL DEHYDROGENASE FAMILY*

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The nucleotide sequence of the structural gene for benzyl alcohol dehydrogenase encoded by TOL plasmid pWW0 of Pseudomonas putida has been determined. Benzyl alcohol dehydrogenase is a member of the long-chain zinc alcohol dehydrogenase family and, like other alcohol dehydrogenases of this family, contains two zinc atoms per subunit. Benzyl alcohol dehydrogenase, while sharing 31% identical residues with horse liver alcohol dehydrogenase, contains several amino acid substitutions near the active site, some of which may be responsible for the substrate specificity of benzyl alcohol dehydrogenase, which oxidizes exclusively aromatic substrates. Benzyl alcohol dehydrogenase also notably lacks the His51 residue of horse liver alcohol dehydrogenase. Contrary to the results obtained with a mutant human liver alcohol dehydrogenase lacking this residue, the concentration and pKₐ of solvent proton acceptors had no effect on the catalytic efficiency of benzyl alcohol dehydrogenase. The electronic nature of substituents on the aromatic ring of the substrate influenced the kₐₚ of the enzyme in low concentrations of external proton acceptor, but not in high concentrations. Product inhibition studies demonstrated that benzyl alcohol dehydrogenase followed a general Ordered Bi Bi kinetic mechanism in low proton acceptor conditions, while following a Theorell-Chance kinetic mechanism at high proton acceptor conditions.

TOL plasmid pWW0 from Pseudomonas putida encodes a set of enzymes responsible for the oxidation of toluene and xylenes. This degradative pathway consists of two parts: an upper pathway that converts toluene and xylenes to their upper pathway that converts toluene and xylenes to their derivatives (Harayama et al., 1989), and a lower pathway that transforms the (substituted) benzoic acids to Krebs cycle intermediates (Harayama and Rekik, 1990). The upper pathway is composed of three enzymes. The first enzyme, xylene monoxygenase (EC 1.14.15.-), is composed of two different subunits encoded by the genes xylA and xylM (Suzuki et al., 1991; Shaw and Harayama, 1992), whereas the second and third enzymes, benzyl alcohol dehydrogenase (EC 1.1.1.90), coded for by xylB and benzaldehyde dehydrogenase (EC 1.2.1.28), coded for by xylC, are each composed of a single polypeptide species. These two dehydrogenases have been extensively characterized (Shaw and Harayama, 1990; Shaw et al., 1992). Benzyl alcohol dehydrogenases have also been found in other bacterial species, and those from Acinetobacter calcoaceticus and P. putida containing TOL plasmid pWW53 have also been purified and characterized (Chalmers and Fewson, 1989; Chalmers et al., 1990, 1991).

NAD/NADP*-dependent alcohol dehydrogenases are classified into four distinct families whose molecular masses are similar in each class. The classical zinc-containing alcohol dehydrogenase family, which is also called the long-chain alcohol dehydrogenase family, comprises liver- and yeast alcohol dehydrogenases and has subunits of about 42 kDa (Jörnvall et al., 1987). The second family comprises only one alcohol dehydrogenase which has recently been discovered from Acetobacter actini. This enzyme has a polypeptide chain of 72 kDa, which is longer than the long-chain alcohol dehydrogenases (Inoue et al., 1989). The short-chain dehydrogenase family contains at least 20 separate enzymes whose subunit sizes are of about 27 kDa (Persson et al., 1991; Ribas de Pouplana et al., 1991; Niedle et al., 1992). Iron-activated alcohol dehydrogenases constitute the last group, and the subunit molecular mass of these enzymes is about 38 kDa (Williamson and Paquin, 1987; Scopes, 1983).

Benzyl alcohol dehydrogenase encoded by TOL plasmid pWW0 is a dimer of identical subunits of 42 kDa (Shaw and Harayama, 1990). The molecular mass of benzyl alcohol dehydrogenase suggests that this enzyme belongs either to the zinc-containing long-chain alcohol dehydrogenase family or to the iron-activated alcohol dehydrogenase family. However, benzyl alcohol dehydrogenase was insensitive to EDTA in contrast to alcohol dehydrogenase I (long-chain type) and to alcohol dehydrogenase II (iron-activated) from Zymomonas mobilis (Scopes, 1983). The determination of N-terminal amino acid sequences of benzyl alcohol dehydrogenase from P. putida (pWW53) showed 26% sequence identity with that of the horse liver enzyme, but 1 Cys residue of the horse liver enzyme essential for the binding of the active-site zinc is not conserved in this sequence (Chalmers et al., 1991). To characterize further benzyl alcohol dehydrogenase from TOL plasmid pWW0, we sequenced its structural gene xylB.

EXPERIMENTAL PROCEDURES

Materials—The coenzymes were purchased from Sigma and were of the highest quality available. The chemical compounds and buffers
were obtained from Fluka AG (Switzerland) and were also of the highest quality available.

**Plasmids and DNA Sequencing**—The source of DNA was either pGSH2833 and pGSH2848 (Harayama et al., 1989). The HindIII fragments of pGSH2848 were subcloned into pUC18 and pUC19. The methods for DNA sequencing with double-stranded DNA have been described previously (Sanger et al., 1977). Universal primers for pUC18/19 as well as 17-19-mer primers complementary to different regions of xylB purchased from Böhringer Mannheim GmbH (Germany) were used for DNA sequencing. Both strands of the xylB gene were sequenced.

**N-terminal Sequencing of Purified Benzyl Alcohol Dehydrogenase**—The method for the purification of benzyl alcohol dehydrogenase has been described previously (Shaw and Harayama, 1990). The protein sequencing was carried out by automated solid-phase Edman degradation using an automatic protein sequenator from Applied Biosystems.

**Nucleotide Sequence Analysis**—The microcomputer-assisted sequence analysis was done with the PC/GENE software package (Intelligenetics) developed by A. Bairoch. The search for proteins exhibiting sequence similarity was done by the method of Lipman and co-workers (Lipman and Pearson, 1988; Wilbur and Lipman, 1988). The protein bank Swiss-Prot compiled by A. Bairoch, the alignment of amino acid sequences was carried out using a computer program, CLUSTAL (Higgins and Sharp, 1988).

**Enzyme Assays**—Enzyme activity was generally measured as described in Shaw and Harayama (1990). The kinetic constants of the dehydrogenase reaction were measured in 20 mM CAPS/NaOH (pH 9.4) containing 100 mM NaCl and 5 mM β-mercaptoethanol (hereafter termed the CAPS/NaCl buffer), and in 100 mM glycine/NaOH (pH 9.4) containing 5 mM β-mercaptoethanol (hereafter termed the glycine buffer). The dependence of the activity of benzyl alcohol dehydrogenase on the concentration (0–100 mM) and pH of formate, acetate, citrate, phosphate, glycollc acid, and glycine, and glycine was measured in the CAPS/NaCl buffer.

The Michaelis-Menten kinetic parameters were determined in these two buffer systems at an enzyme concentration of 13 nm. In the assays performed on the dehydrogenation reaction, the concentrations of benzyl alcohol and NAD were varied from 0 to 400 μM, while the benzaldehyde reduction reaction (the reverse reaction of benzyl alcohol dehydrogenase), the concentrations of benzaldehyde and NADH were varied from 10 to 100 μM and 10 to 360 μM, respectively.

The product inhibition studies were performed in either the glycine or CAPS/NaCl buffer containing varying concentrations of one of the reaction products (NADH or benzaldehyde), varying concentrations of one of the substrates (NAD+ or benzyl alcohol), and a constant concentration of the other substrate. The concentration of this constant substrate was maintained close to its measured Km value, either 200 μM for NAD+ or 150 μM for benzyl alcohol, while the concentration of benzaldehyde was varied from 10 to 100 μM and the concentration of NADH was varied from 10 to 200 μM. The rate of the reactions was followed by measuring the rate of NADH production at 340 nm in an Uvikon 940 double-beam spectrophotometer.

All kinetic measurements were performed at least five times, and the mean value was used for the subsequent calculations.

**Assays of the Zinc Content of Benzyl Alcohol Dehydrogenase**—The purified enzyme (1 mg in 200 μl) was dialyzed at 4 °C against one of the following buffers: 100 mM Tris-Cl (pH 6.5), 100 mM phosphate (pH 7.4), and 100 mM sodium acetate buffer (pH 5.5), with and without 5 mM benzyl alcohol. These buffers were prepared in 1 liter of Milli-Q water (Millipore) in 1-liter plastic bottles, which had been previously washed with concentrated HCl and HNO3, before being extensively washed with Milli-Q water. The dialysis against 100 mM sodium acetate buffer (pH 5.5) containing 5 mM benzyl alcohol caused the least inactivation of the enzyme. On the basis of these results, aliquots of purified benzyl alcohol dehydrogenase (generally 5–10 mg in 3 ml) were dialyzed twice against 1 liter of 100 mM sodium acetate buffer (pH 5.5) containing 5 mM benzyl alcohol, at 4 °C under nitrogen atmosphere, for periods generally greater than 72 h. The aliquots were transferred to washed PVC tubes, and the zinc content of the enzyme solutions and of the exterior buffer were determined by atomic absorption at 213.9 nm of an air-acetylene flame using an atomic absorption spectrometer (Pye Uvicam FP-9, United Kingdom).

**RESULTS**

**Nucleotide Sequence of xylB**

The location of xylB has previously been determined by subcloning and transposon mutagenesis (Harayama et al., 1986, 1989). The complete DNA sequence of 1940-base pair long DNA of the TOL plasmid pWW0 containing xylB was determined (Fig. 1). The open reading frame analysis was preceded by GAGGGGAG which may constitute ribosome-binding sites. The molecular weight of the deduced xylB product is 38,510, which is in agreement with the result in maxicells (Harayama et al., 1989) and with the molecular mass of purified benzyl alcohol dehydrogenase (Shaw and Harayama, 1990). The N-terminal sequence of purified benzyl alcohol dehydrogenase was Met-Xaa-Ile-Lys-Ala-Ala-Ile-Val. This result supports our assignment of the initiation codon for xylB.

**Similarity of xylB with Zinc-containing Long-chain Alcohol Dehydrogenases**

A scan of the protein bank Swiss-Prot detected a similarity between the amino acid sequence of benzyl alcohol dehydrogenase and those of zinc-containing alcohol dehydrogenases. The zinc-containing dehydrogenases are classified into five groups (Jornvall et al., 1987): dimeric mammalian/plant alcohol dehydrogenases, bacterial dehydrogenases, tetrameric yeast alcohol dehydrogenases, tetrameric poliovirus alcohol dehydrogenases, and threonine dehydrogenases. Mammalian/plant alcohol dehydrogenases are further divided into three subgroups, class I, II, and III (Eklund et al., 1990). Subunit types α, β, and γ that constitute the isoenzymes of human alcohol dehydrogenase and the E subunit of horse liver alcohol dehydrogenase belong to class I. Human alcohol dehydrogenase φ subunit and two isoenzymes of maize alcohol dehydrogenase are class II enzymes. Class III contains the χ subunit of human alcohol dehydrogenase, avian alcohol dehydrogenase, and a lens crystalline. The crystalline, although structurally similar to class III dehydrogenases, does not contain zinc (Barras et al., 1989). When the sequence similarities between benzyl alcohol dehydrogenase and these five groups of long-chain zinc-containing alcohol dehydrogenases were compared, it was clear that benzyl alcohol dehydrogenase is more related to the group of mammalian/plant alcohol dehydrogenases than to other groups of alcohol dehydrogenases. The evolutionary distances between benzyl alcohol dehydrogenase and mammalian/plant dehydrogenases was, however, larger than those between class I, II, and III of mammalian/plant dehydrogenases. Notably, 31% of the amino acids in the benzyl alcohol dehydrogenase sequence are identical with those of the horse liver alcohol dehydrogenase E.

**Zinc Content of Benzyl Alcohol Dehydrogenase**

The deduced sequence of benzyl alcohol dehydrogenase strongly suggested the presence of protein-bound zinc. In order to determine the zinc content of the purified protein, we first determined conditions of dialysis which minimize the inactivation of the enzyme. When the purified benzyl alcohol dehydrogenase was dialyzed under N2 at 4 °C in 100 mM acetate buffer (pH 5.5) containing 5 mM benzyl alcohol, the enzyme conserved its initial activity after the dialysis for 12 h. The results of the atomic absorption spectrometry of benzyl alcohol dehydrogenase under these conditions were in agreement with the results in the literature: about 2.7 of the covalent zinc/mol of enzyme. The purified benzyl alcohol dehydrogenase was thus classified as zinc-containing alcohol dehydrogenase.

**Kinetic Studies on Benzyl Alcohol Dehydrogenase**

The methods for the purification of benzyl alcohol dehydrogenase have been described previously (Shaw and Harayama, 1990). The protein sequencing was carried out by automated solid-phase Edman degradation using an automatic protein sequenator from Applied Biosystems.

**Nucleotide Sequence Library with accession number M94184.**

1 The DNA sequence of xylB has been submitted to the EMBL Nucleotide Sequence Library with accession number M94184.

2 The abbreviations used are: CAPS, 3-cyclohexylammonium 1-propanesulfonic acid.
Influence of the Concentration and pH of External Proton Acceptors on Benzyl Alcohol Dehydrogenase Activity

In a preliminary study, it was determined that the benzyl alcohol dehydrogenase activity was not influenced by varying concentrations of CAPS buffer (data not shown), but it was found that the activity was dependent on salt concentrations (Fig. 2). The activity of benzyl alcohol dehydrogenase nearly doubled when the concentrations of NaCl, KCl, NH₄Cl, KBr, and KNO₃ in 20 mM CAPS (pH 9.4) increased from 0 to 25 mM. However, benzyl alcohol dehydrogenase activity was independent of the salt concentration when the concentration is between 75 and 400 mM (data not shown). We therefore used 20 mM CAPS/NaOH buffer containing 100 mM NaCl and 5 mM β-mercaptoethanol as low proton acceptor conditions for the benzyl alcohol dehydrogenase dialysis.

Fig. 1. Nucleotide sequence of xylB. The nucleotide sequence of the 1940-base pair region of the TOL plasmid pWWO containing alcohol dehydrogenase is divided in these conditions indicated the presence of 1.9 ± 0.5 atoms of zinc/subunit of benzyl alcohol dehydrogenase. This confirmed the presence of two atoms of zinc/subunit. This was expected from the sequence data, which indicated that the residues Cys90, His61, and Cys169 which bind the catalytic zinc atom, and the residues Cys89, Cys97, and Cys104 which bind a noncatalytic zinc atom of horse liver alcohol dehydrogenase were conserved in benzyl alcohol dehydrogenase. The dialysis of benzyl alcohol dehydrogenase in the presence of 5 mM EDTA or 2,6-pyridine dicarboxylic acid did not reduce the enzyme activity significantly, but dialysis against 100 mM acetate buffer (pH 5.5) containing 5 mM benzyl alcohol and 5 mM o-phenanthroline resulted in the precipitation of the protein, with the loss of over 95% of its initial activity. Thus, o-phenanthroline, but not other chelators, may remove enzyme-bound zinc atoms.

Although all the zinc-containing long-chain alcohol dehydrogenases share the same chemical reaction, namely NAD+/NADP+-dependent oxidation of alcohol to aldehyde, many of them exhibit functional heterogeneity in the binding of coenzymes, inhibitors, and substrates, or the subunit interactions. Benzyl alcohol dehydrogenase has several peculiar functional characteristics: substrate specificity highly specific toward aromatic compounds, pH optima at an alkaline pH for its forward reaction and an acid pH for its reverse reaction, and broad substrate specificity toward substituted benzyl alcohols (Shaw and Hayrana, 1990; Shaw et al., 1992).

We are interested in investigating these characteristics on the basis of the tertiary structure of horse liver alcohol dehydrogenase (Eklund et al., 1976, 1982a, 1982b; Plapp et al., 1978, 1990). In this study, we focused on the fact that benzyl alcohol dehydrogenase lacks the amino acid residue corresponding to His54 of the horse isoenzyme. In horse liver alcohol dehydrogenase, His54 has been proposed to act as a general base that abstracts a proton from the alcohol substrate through a proton relay system connecting: zinc atom-oxidized substrate-Ser156, coenzyme ribose-His51 (Eklund et al., 1976). Isolation and characterization of a mutant HisS1-Gln of human liver alcohol dehydrogenase supported this proposal (Hurley et al., 1990). His54 is replaced by Val64 in benzyl alcohol dehydrogenase, and by Ser in human class II alcohol dehydrogenases. In these enzymes, the proton-relay system may be rearranged and connected to a water molecule or another general base. If this is the case, the enzymatic activity of benzyl alcohol dehydrogenase is expected to depend, to a certain extent, on the concentration of an external proton acceptor, and on its pKa, as was observed for a His51-Gln mutant of human liver alcohol dehydrogenase (Hurley et al., 1990).

10844

Kinetic Studies on Benzyl Alcohol Dehydrogenase

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xyB (residues 187–1287) is presented. The amino acid sequence of benzyl alcohol dehydrogenase is also shown. The putative Shine-Dalgarno sequence of xylB is underlined.

The nucleotide sequence of the 1940-base pair region of the TOL plasmid pWWO containing
Kinetic Studies on Benzyl Alcohol Dehydrogenase

The Michaelis-Menten kinetic parameters of the forward and reverse reactions of benzyl alcohol dehydrogenase in the CAPS/NaCl and glycine buffers were determined by varying the concentrations of both substrates (Table I and II). The family of Lineweaver-Burk plots obtained at different substrate concentrations intersect at a single point to the left of the 1/v axis (results not shown). This result reveals the presence of a central ternary complex. Among the different classes of kinetic mechanisms involving two substrates and two products (Bi-Bi systems), the ping-pong (in which the first substrate bound is released before the binding of the second substrate), and the general random (in which the order of binding of the two substrates is random) mechanisms, which do not contain a common intersection point on the Lineweaver-Burk plot, could be eliminated from the possibilities, and two other alternatives, the ordered (in which one substrate must be bound before the second) and the rapid equilibrium random kinetic mechanisms remained as possibilities. As described below, the rapid equilibrium random kinetic mechanism was subsequently excluded from the possibilities.

The global rate equation of the Ordered Bi-Bi kinetic

**TABLE I**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Value (µM)</th>
<th>S.E. (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate</td>
<td>155 ± 8</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
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<td></td>
</tr>
<tr>
<td>Phosphate</td>
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</tbody>
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**TABLE II**

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<thead>
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</tbody>
</table>

**Influence of the Buffer Conditions on the Michaelis-Menten Kinetic Parameters for the Dehydrogenation Reaction**

Benzyl alcohol dehydrogenase catalyzes the oxidation of benzyl alcohol to benzaldehyde with the concomitant reduction of NAD⁺. Conditions described under “Experimental Procedures.” Kₐ represents the dissociation constant of the first substrate bound from its enzyme-substrate complex. The values after the ± indicate the S.E. expected from linear regression analysis.

**TABLE III**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Vₘₐₓ (µmol min⁻¹ mg⁻¹)</th>
<th>Kₐ (µM)</th>
<th>Kₛ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mM glycine + 20 mM CAPS/NaOH</td>
<td>320 ± 15</td>
<td>290 ± 15</td>
<td></td>
</tr>
<tr>
<td>200 mM CAPS/NaOH + 100 mM NaCl</td>
<td>290 ± 15</td>
<td>290 ± 15</td>
<td></td>
</tr>
</tbody>
</table>

**Kinetic Parameters for the Dehydrogenation Reaction**

Benzyl alcohol dehydrogenase catalyzes the reduction of benzaldehyde to benzyl alcohol with the concomitant oxidation of NADH. Conditions described under “Experimental Procedures.” In this paper, the same nomenclature of the kinetic constants was used for the forward and reverse reactions. In the forward reaction, the first and second substrates bound to the enzyme are called A and B, respectively, while the first and second product released from the enzyme are called P and Q, respectively (Fig. 4). In the reverse reaction, the first and second substrate bound to the enzyme is Q and P by this definition. Therefore, Kₛ represents the dissociation constant of the first substrate bound in the reverse direction from the enzyme-substrate complex. The values after the ± indicate the S.E. expected from linear regression analysis.

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mechanisms, which can be further classified as general ordered, rapid equilibrium ordered, and Theorell-Chance ordered is described in all three cases by the same kinetic equation (Cleland, 1970). In the following discussion, the nomenclature of Cleland (1970) will be employed. In this nomenclature, \( K_a \) is the dissociation constant of substrate A (=\( a_1/k_1 \)), which is the first substrate bound to the enzyme. \( K_m \) and \( K_{nb} \) are the Michaelis-Menten constants for substrates A and B, respectively, and \( V_{max} \) corresponds to the \( V_{max} \) of the forward reaction. \( P \) and \( Q \) are the reaction products.

The basic Michaelis-Menten kinetic parameters of the forward reaction in the CAPS/NaCl and glycine buffers were essentially unchanged. The Michaelis-Menten parameters for benzyl alcohol dehydrogenase functioning in the reverse direction, i.e. catalyzing the reduction of benzaldehyde to benzyl alcohol with the concomitant oxidation of NADH to NAD\(^+\), were also determined. The pH optimum for this reaction is at pH 5.7, but there is sufficient residual activity at pH 9.4 to permit the measurement of the different kinetic constants (see Table II). As can be seen in Table II, the \( K_m \) value of NADH was significantly influenced by the buffer conditions, and was four times lower in the CAPS/NaCl buffer.

**Product Inhibition of Benzyl Alcohol Dehydrogenase in the CAPS/NaCl Buffer**

The products of the forward reaction of benzyl alcohol dehydrogenase, NADH, and benzaldehyde inhibited the enzymatic reaction. The results of the product inhibition in the CAPS/NaCl buffer are shown in Table III. From the rate equation of a general ordered Bi-Bi kinetic mechanism which describes the inhibition by Q (Segel, 1975), this product is expected to be a competitive inhibitor of A.

In the CAPS/NaCl buffer, the only competitive inhibition pattern observed was between NADH and NAD\(^+\) (see Table III). It was therefore concluded that A is NAD\(^+\), and Q is NADH, and as a consequence, B is benzyl alcohol, and P is benzaldehyde. This sequence of substrate addition and product release is the same as that observed for various other alcohol dehydrogenases, notably the yeast (Plapp et al., 1990), horse (Theorell and Chance, 1951), and human alcohol dehydrogenases (Yin et al., 1984). The inhibition pattern in Table III eliminates the rapid equilibrium Orderd Bi-Bi mechanism from the possibilities, since in this mechanism, the products of the reaction should either be competitive toward both substrates (if there is no abortive complex formation) or benzaldehyde would be a competitive and noncompetitive inhibitor, and NADH a noncompetitive and competitive inhibitor towards benzaldehyde and NAD\(^+\), respectively (Segel, 1975). This inhibition pattern also eliminates the rapid equilibrium random Bi-Bi kinetic mechanism, since in this mechanism, the product (P) would be a competitive inhibitor towards both substrates A and B, while the reaction product (Q) would be a competitive inhibitor toward A, but a noncompetitive inhibitor toward B.

**NADH Inhibition**—As mentioned above, NADH is a competitive inhibitor toward NAD\(^+\). In the Lineweaver-Burk plot at different concentrations of NADH, the abscissa intercepts were constant. According to the rate equation of a general Ordered Bi-Bi kinetic mechanism in the presence of Q (NADH), the slopes of the Lineweaver-Burk plots would increase linearly with increasing [Q]. The replot of the slope of the Lineweaver-Burk plot against [Q] gave a straight line with an abscissa intercept \( K_m \), which was determined from the kinetics of the reverse reaction of benzyl alcohol dehydrogenase in CAPS/NaCl buffer to be 125 \( \mu M \) (Table II). Using the \( K_m \) and \( K_q \) values thus determined, the value of \( K_{nb} \), which corresponds to the dissociation constant of the EQB abortive complex, was calculated from the rate equations described by Segel (1975) to be \( \approx \); the EQB complex does not form in these buffer conditions.

When the concentrations of Q and B (benzyl alcohol) were varied and that of A was held constant, the Lineweaver-Burk replot was nonlinear and is described by a hyperbola. This nonlinearity of \( 1/v \) as a function of \( 1/[B]\), which is generally observed when the concentrations of Q and B are varied, results from the fact that the slope and y intercept of the reciprocal plots are function of both [B] and [Q]. When the calculated values of \( V_{max}, K_m, K_{nb} \), and \( K_q \) were introduced into the rate equation of the general Ordered Bi-Bi kinetic mechanism, the observed inhibition pattern could be reproduced.

**Benzaldehyde Inhibition**—When the concentration of NAD\(^+\) (A) and benzaldehyde (P) were varied in the presence of a constant concentration of benzyl alcohol (B), the slope of the Lineweaver-Burk plot increased linearly with increased P. When the slope values were replotted against P, the slope of the slope replot corresponds to \( (1/V_{max}) \times (K_m \times [B]) \times (K_{nb} / (K_q \times [B])) \). The abscissa intercept point of this replot \( (K_m \text{ in Table III}) \) corresponds to \( (1/v_{max}) \times (K_m + (K_q \times [B]/[B])) \times ((K_m \times [B]) / (K_q \times [B])). \) These relationships were used to confirm the values of the kinetic constants previously determined.

The replot of the values of the ordinate intercept points of the Lineweaver-Burk plot as a function of the concentration of P was parabolic. This result indicates the formation of an abortive EAP complex, which is formed when benzyl alcohol dehydrogenase binds NAD\(^+\) normally, but subsequently binds benzaldehyde rather than benzyl alcohol. The \( K_p \) value which corresponds to the dissociation constant of benzaldehyde from the abortive complex EAP was determined to be 50 \( \mu M \) (Table IV).

When benzaldehyde (P) and benzyl alcohol (B) were varied in the presence of a constant concentration of NAD\(^+\) (A), the ordinate intercept points of the Lineweaver-Burk plot increased linearly with increased [P], with a theoretical slope corresponding to \( (1/V_{max}) \times (1/K_q). \) This relationship permitted the calculation of \( K_q \), which was calculated to be 229.5 \( \mu M \). The parabolic shape of the replot of the slope of the Lineweaver-Burk plot against P could be reproduced when

### Table III

**Product inhibition of benzyl alcohol dehydrogenase by benzaldehyde and NADH in CAPS/NaCl buffer**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Varied substrate</th>
<th>Constant substrate</th>
<th>Inhibition</th>
<th>Slope replot</th>
<th>Ordinate intercept replot</th>
<th>( K_m )</th>
<th>( K_q )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzaldehyde</td>
<td>NAD(^+)</td>
<td>Benzyl alcohol</td>
<td>Noncompetitive (MT)*</td>
<td>Parabolic</td>
<td>Linear</td>
<td>423</td>
<td></td>
</tr>
<tr>
<td>NADH</td>
<td>Benzyl alcohol</td>
<td>NAD(^+)</td>
<td>Noncompetitive</td>
<td>Linear</td>
<td>Parabolic</td>
<td>265</td>
<td></td>
</tr>
<tr>
<td>NADH</td>
<td>Benzyl alcohol</td>
<td>NAD(^+)</td>
<td>Noncompetitive nonlinear</td>
<td>Linear</td>
<td>Constant</td>
<td>120</td>
<td></td>
</tr>
</tbody>
</table>

*MT: mixed type. The Lineweaver-Burk plots do not intersect at a single point.*
the values of $K_{\text{app}}/(K_{\text{app}}K_{\text{mp}})$ and $K_{\text{ip}}$ determined above were introduced into the equation describing this parabolic curve.

**Product Inhibition of Benzyl Alcohol Dehydrogenase in Glycine Buffer**

The inhibition by the reaction products on the activity of benzyl alcohol dehydrogenase were also examined in the glycine buffer (see Table V). The analysis of these inhibition patterns was based on the substrate binding and product release orders determined for benzyl alcohol dehydrogenase in the CAPS/NaCl buffer. The inhibition patterns were almost identical to those in the CAPS/NaCl buffer, with the exception of the inhibition produced by benzaldehyde (P) against benzyl alcohol (B) with the concentration of NAD$^+$ (A) held constant. In this particular case, benzaldehyde is a competitive inhibitor of benzyl alcohol, while in the CAPS/NaCl buffer the inhibition was mixed-type noncompetitive. This difference could allow the expression of the kinetic results obtained in the glycine buffer by the simpler initial rate equation describing a Theorell-Chance Bi-Bi mechanism (Cleland, 1970; Segel, 1975). The Ordered Bi-Bi mechanism, observed in the CAPS/NaCl buffer, therefore reduces to a Theorell-Chance Ordered Bi-Bi mechanism in the glycine buffer (Fig. 4). The binding and release order in the glycine buffer cannot be affirmed on the basis of the inhibition pattern in the glycine buffer, but we assume that the order remains the same, since the order of binding and release is the same for all alcohol dehydrogenases, and since it is unlikely that a change in the buffer system leads to a different order of substrate binding and product release. The implications of the difference in the two kinetic mechanisms will be discussed below.

**Product Inhibition by NADH**—The presence of varying concentrations of NADH (Q) with varying concentrations of NAD$^+$ (A), with benzyl alcohol (B) constant, in the glycine buffer gave rise to a competitive inhibition, as was also the case in the CAPS/NaCl buffer. This inhibition of benzyl alcohol dehydrogenase by NADH in glycine buffer could be described by the Theorell-Chance global rate equation, which is actually the same equation as in the Ordered Bi-Bi mechanism. Thus, the abscissa intercept of the replot of the slope of the Lineweaver-Burk plot against [Q] ($K_q$ in Table V) is given by $-K_{\text{ip}}/(1 + [B]/K_{\text{ip}})$. Using the value of $K_{\text{ip}}$ (75 $\mu$M) determined by the kinetic analysis of the reverse reaction of benzyl alcohol dehydrogenase in glycine buffer gave rise to a value of 1 + [B]/$K_{\text{ip}}$ of 0.58, although this value should be greater than 1. If it is assumed that the value of $K_{\text{ip}}$ is infinitely large, and as a consequence that the [B]/$K_{\text{ip}}$ term is essentially null, the value of $K_q$ obtained from the inhibition by NADH is 129 $\mu$M.

The product inhibition pattern in the presence of varying concentrations of NADH and benzyl alcohol (Q versus B) gave rise to nonlinear relationship between 1/$\nu$ and 1/[B]. The observed curves could be reproduced when the calculated values of $V_{\text{app}}$, $K_{\text{mB}}$, $K_{\text{mQ}}$ in Table I were introduced into the Theorell-Chance rate equation and $K_q$ was assumed to be 129 $\mu$M rather than 75 $\mu$M.

**Product Inhibition by Benzaldehyde**—The inhibition pattern produced by benzaldehyde (P) in the glycine buffer in the presence of varying concentrations of benzyl alcohol (with NAD$^+$ constant) could be reproduced when the calculated kinetic constants were introduced into the rate equation of a Theorell-Chance Ordered Bi-Bi mechanism with the possible formation of the abortive EAP ternary complex. When benzaldehyde (P) and NAD$^+$ (A) are varied, with a constant concentration of benzyl alcohol (B), the replot of the slope of the Lineweaver-Burk plot against the concentration of P is linear, with a slope of the replot corresponding to $(1/V_{\text{app}}) \times (K_qK_{\text{mp}}/[B]) \times (1/K_{\text{ip}})$ and with the abscissa intercept point ($K_{\text{ip}}$ in Table V) corresponding to $-K_{\text{ip}}(1 + (K_{\text{mp}}[B])/(K_qK_{\text{mp}}))$. The value of $K_{\text{ip}}$ was calculated to be 5.9 $\mu$M. The replot of the ordinate intercept points of the Lineweaver-Burk plot as a function of the concentration of P gave rise to a parabolic curve. By searching for the value of $K_{\text{ip}}$ which best fits this curve, the value of $K_{\text{ip}}$ could be determined (see Table IV). $K_{\text{ip}}$ corresponds to the dissociation constant of the EAP complex to free P (benzaldehyde) and EA complex, and as a consequence is a reflection of the facility with which benzalcohol dehydrogenase binds benzaldehyde in the active site at the place of benzyl alcohol. The values of $K_{\text{ip}}$ shown in Table IV indicate that the dissociation of the EAP complex is independent of the buffer conditions.

**Influence of the Electronic Nature of Substituents on the Aromatic Ring of the Substrate on the Rate of the Benzyl Alcohol Dehydrogenase-catalyzed Reaction in CAPS/NaCl Buffer**

The effects of substitution on the aromatic ring on the rate of a reaction occurring on another substituent on the aromatic ring can be described by the free-energy relationship of Hammett (Hammert, 1940). The influence of the substitution on the aromatic ring of benzyl alcohols in the glycine buffer has already been determined (Shaw et al., 1992), and it has been noted that the electronic nature of the substituents on the aromatic ring of benzyl alcohol have no effect on the $k_{\text{cat}}/K_m$ of the benzyl alcohol dehydrogenase-catalyzed reaction. The effect of substitution on the aromatic ring of benzyl alcohol

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**Table IV**

Values of the kinetic constants deduced from product inhibition rate equations

Calculated as described in the text on the basis of the rate equations described by Segel (1975). Values after the ± indicate the S. D. expected after analysis by linear regression.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>100 mM glycine/NaOH</th>
<th>20 mM CAPS/NaOH + 100 mM NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{\text{ip}}$ ($\mu$M)</td>
<td>50 ± 7</td>
<td>50 ± 9</td>
</tr>
<tr>
<td>$K_{\text{ip}}$ ($\mu$M)</td>
<td>6 ± 4</td>
<td>230 ± 35</td>
</tr>
<tr>
<td>$K_{\text{ip}}$ ($\mu$M)</td>
<td>∞</td>
<td>∞</td>
</tr>
</tbody>
</table>

**Table V**

Product inhibition of benzyl alcohol dehydrogenase by benzaldehyde and NADH in glycine buffer

Determined in 100 mM glycine/NaOH (pH 9.4) containing 5 mM β-mercaptoethanol.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Varied substrate</th>
<th>Constant substrate</th>
<th>Inhibition</th>
<th>Slope replot</th>
<th>Ordinate intercept replot</th>
<th>$K_q$</th>
<th>$K_{\text{ip}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzaldehyde</td>
<td>Benzyl alcohol</td>
<td>NAD$^+$</td>
<td>Competitive</td>
<td>Parabolic</td>
<td>Constant</td>
<td>$\mu$M</td>
<td></td>
</tr>
<tr>
<td>NAD$^+$</td>
<td>Benzyl alcohol</td>
<td>NAD$^+$</td>
<td>Noncompetitive</td>
<td>Linear</td>
<td>Parabolic</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>NAD$^+$</td>
<td>Benzyl alcohol</td>
<td>Benzyll alcohol</td>
<td>Noncompetitive nonlinear</td>
<td>Linear</td>
<td>Constant</td>
<td>130</td>
<td></td>
</tr>
</tbody>
</table>
**Ordering Studies on Benzyl Alcohol Dehydrogenase**

**Substrate Binding**—The substrate-binding pocket of horse liver alcohol dehydrogenase is formed by a deep hydrophobic cleft lined by some 12 amino acids (Jornvall, 1987). The zinc ligands (Cys, His) and residues Ser48, Phe93, Phe140, and Leu141 constitute the inner part of the substrate pocket. The inner part of the substrate cleft in benzyl alcohol dehydrogenase may be smaller than that of the horse enzyme, because both Ser48 and Leu141 are replaced by larger residues, Thr48 and Phe140 respectively. In the middle part of the substrate-binding pocket, Leu67 in the horse enzyme is replaced by Tyr51 in benzyl alcohol dehydrogenase. Four aromatic residues, Phe67, Phe136, and Tyr51, are therefore clustered in this region of benzyl alcohol dehydrogenase forming an internal hydrophobic core. Contrary to the substitutions mentioned above, Val284 in the horse enzyme is replaced by smaller Ala296 in benzyl alcohol dehydrogenase. A substitution from Phe110 of the horse enzyme to Ser103 of benzyl alcohol dehydrogenase may make the mouth of the pocket more large.

In class II and III subunits of human alcohol dehydrogenases, the inner parts of the substrate-binding pockets are narrower, and their affinities toward ethanol are smaller than those of class I enzymes (Ekland et al., 1990). On the other hand, the affinities to larger aliphatic alcohols are higher in the human class II enzymes than in the class I enzymes. The class II enzyme, with its narrow hydrophobic substrate-binding site, is apparently adapted to larger substrates. A similar situation occurs in benzyl alcohol dehydrogenase. The substrate-binding site of this enzyme is narrower than in the class I enzymes, and the rate of ethanol does not accept ethanol but does accept larger aromatic alcohol substrates. This tendency, namely that narrower substrate-binding sites exhibit a preference for larger substrates, is not universal, since the narrow substrate-binding pocket of yeast alcohol dehydrogenase exhibits a strong specificity for ethanol. In the latter enzyme, genetic studies have demonstrated that Thr48 (numbering according to horse liver alcohol dehydrogenase) makes this enzyme specific toward ethanol, and a mutant enzyme Thr48-Ser with a larger substrate-binding site, exhibits an increased activity towards larger aliphatic alcohols (Plapp et al., 1990). It is interesting to note that although this residue is Thr48 in benzyl alcohol dehydrogenase, the enzyme displays no detectable activity or affinity toward ethanol.

**Coenzyme Binding**—Several residues are involved in the cofactor binding in horse liver alcohol dehydrogenase. Thr178 and Val200 of this enzyme which interact with the nicotinamide ring are conserved in benzyl alcohol dehydrogenase. Polypeptide-backbone nitrogen atoms of residues Gly80 and Gly80 of the horse liver enzyme could interact with an oxygen of the phosphate proximal to the nicotinamide group. The residue corresponding to Gly80 is conserved in benzyl alcohol dehydrogenase while the residue corresponding to Gly80 is substituted (Ala79). Leu285 of horse liver alcohol dehydrogenase makes van der Waals contacts with pyrophosphate oxygen atoms of NAD+. Benzyl alcohol dehydrogenase contains Ser353 at this position which may or may not contact with NAD+.

Benzyl alcohol dehydrogenase also contains Asp218, which corresponds to the Asp215 residue of the horse liver alcohol dehydrogenase, which has been shown to be important in determining the specificity of the dehydrogenase toward NAD+ rather than NADP+ (Fan et al., 1991). It has been determined that benzyl alcohol dehydrogenase is far less...
active in the presence of NADP+ than in the presence of NAD+, with $K_m$ values of 1.5 mm and 47.6 μM, respectively. Benzyl alcohol dehydrogenase shares another property with horse liver alcohol dehydrogenase; that of transferring hydride to the pro-R side of the nicotinamide ring of NAD+ (Shaw and Harayama, 1990; You, 1985).

In horse liver alcohol dehydrogenase, Arg47 binds the pyrophosphate of the NAD+ coenzyme (Eklund et al., 1981). Position 47 in many enzymes is Arg, but substitution to His is frequently observed. The substitution from Arg to His at position 47, observed in class II, class III, and yeast alcohol dehydrogenases, is expected to weaken the binding of NADH at pH values at which the histidine residue is not protonated. In the zinc-containing alcohol dehydrogenases characterized so far, the last step is rate-limiting (Wratten and Cleland, 1965). The decrease in the affinity to NAD(P)H may increase the $k_{cat}$ value of the enzymes (Eklund et al., 1990). His47 in the yeast isoenzyme I was substituted with an Arg by the site-directed mutagenesis. The substitution decreased dissociation constants 4-fold for NAD+ and 2-fold for NADH (Gould and Plapp, 1990). In human β enzymes, the magnitudes of these effects observed in p2-Oriental and p2-Bern variants are even more striking (Jörnvall et al., 1984). In human β3β3 alcohol dehydrogenase in which the amino acid at position 47 was changed by site-directed mutagenesis, the more basic the amino acid residue at that position, the higher the affinity for NAD+ (Hurley et al., 1990). In benzyl alcohol dehydrogenase, the corresponding position is His. The relatively high $k_{cat}$ value of this enzyme may partly be due to the presence of this His residue. Arg47 of horse liver alcohol dehydrogenase is also the NADH phosphate-binding site. This residue is Lys36 in benzyl alcohol dehydrogenase. The residues Ser48 and His51 which bind the nicotinamide ring and the substrate in horse liver alcohol dehydrogenase are substituted to Thr45 and Val55 in benzyl alcohol dehydrogenase. The Ser to Thr substitution at Ser48 is also found in the human enzymes (with the exception of human γ1 and γ2 class I enzymes), while benzyl alcohol dehydrogenase is the only enzyme to have a hydrophobic non-polar residue at position 51, the human and horse enzyme all having a basic residue at this position (except human class II enzymes).

### Table VI

<table>
<thead>
<tr>
<th>Rate constants of benzyl alcohol dehydrogenase in glycine and CAPS/NaCl buffer</th>
<th>Rate constants</th>
<th>100 mm glycine/NaOH (pH 9.4)</th>
<th>20 mM CAPS (pH 9.4) + 100 mM NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_1 (\mu M^{-1} s^{-1})$</td>
<td>1.1</td>
<td>$k_3 (\mu M^{-1} s^{-1})$</td>
<td>0.9</td>
</tr>
<tr>
<td>$k_2 (\mu M^{-1} s^{-1})$</td>
<td>233</td>
<td>$k_4 (\mu M^{-1} s^{-1})$</td>
<td>292</td>
</tr>
<tr>
<td>$k_3 (\mu M^{-1} s^{-1})$</td>
<td>1.5</td>
<td>$k_5 (\mu M^{-1} s^{-1})$</td>
<td>ND*</td>
</tr>
<tr>
<td>$k_4 (\mu M^{-1} s^{-1})$</td>
<td>52</td>
<td>$k_6 (\mu M^{-1} s^{-1})$</td>
<td>ND</td>
</tr>
<tr>
<td>$k_5 (\mu M^{-1} s^{-1})$</td>
<td>223</td>
<td>$k_7 (\mu M^{-1} s^{-1})$</td>
<td>ND</td>
</tr>
<tr>
<td>$k_6 (\mu M^{-1} s^{-1})$</td>
<td>1.7</td>
<td>$k_8 (\mu M^{-1} s^{-1})$</td>
<td>7.2</td>
</tr>
</tbody>
</table>

* ND, not determined.

In the CAPS/NaCl buffer, the inhibition patterns resembled those produced by a general Ordered Bi-Bi mechanism, while in the glycine buffer the mechanism was apparently a Theorell-Chance Ordered Bi-Bi. The mechanism has been called a hit-and-run mechanism, since the production of P after the binding of B is very rapid, compared to the other rates in the reaction pathway, and the concentration of the central ternary complexes EAB and EQP is essentially zero (Fig. 4). The Ordered Bi-Bi kinetic mechanism reduces to a Theorell-Chance kinetic mechanism when the constants $k_B$ (defined as $(k_0 + k_0)/k_3$) and $K_a$ (defined as $(k_1 + k_2)/k_3$) in the Ordered Bi-Bi kinetic mechanism are very large compared to the others. These conditions are fulfilled when $k_0 >> k_3$, $k_0 >> k_4$, $k_1 >> k_2$, and $k_1 >> k_2$. The fact that the Theorell-Chance kinetic mechanism did not describe benzyl alcohol dehydrogenase in the CAPS/NaCl buffer indicates that either the value of $k_3$ or $k_2$ is significantly reduced or that the isomerization step EAB→EQP (Fig. 4) is significantly slowed down in the CAPS/NaCl buffer.

The Michaelis-Menten constants determined for benzyl alcohol dehydrogenase in both directions permitted the calculation of a certain number of the rate constants of the reaction equations in the two buffer systems. The rate constants of the Theorell-Chance rate equation in the glycine buffer were all calculated (see Table VI). From the $k_1$ and $k_2$ values thus obtained, the $K_v$ value ($k_0/k_3$) was calculated to be 4.3 μM, which is in agreement with the $K_v$ value determined experimentally (5.9 μM in Table IV). In the Ordered Bi-Bi mechanism observed in the CAPS/NaCl buffer, the only rate constants that allow a direct comparison with the same constants in the Theorell-Chance mechanism were those for the binding of the coenzyme. A comparison of the rate constants concerning the binding of coenzyme reveals that the $k_1$ and $k_2$, obtained in the two buffer systems were very similar and that, as a consequence, the binding of NAD+ is not particularly affected by the buffer conditions. The binding of NADH is affected by the nature of the buffer, the rates of binding and release of NADH to the free enzyme increasing 4-fold in the CAPS/NaCl buffer. It can also be observed that the slowest step in the kinetic mechanism in the glycine buffer is the release of NADH ($k_1$), which has also been observed in a large number of alcohol dehydrogenases (Brändén et al., 1975). The calculation of the other rate constants gave rise to negative values for $k_4$, $k_5$, and $k_7$. However, if the rate constants calculated for a given mechanism have negative values or are smaller than the maximal velocity, it is likely that stable enzyme forms are isomerizing in at least partially rate-limiting steps (Rudolph, 1980), which would appear to be the case for benzyl alcohol dehydrogenase in the CAPS/NaCl buffer.

In conclusion, we demonstrated that benzyl alcohol dehydrogenase, lacking the His51 residue, possesses a system for the removal of a H+ from the active site whose efficiency is not influenced by the concentration or $pK_a$ of external bases. We also have demonstrated that the rate of conversion of the EAB complex to the EQB complex is diminished in the absence of glycine and that this transformation step is involved in the development of a charge on the substrate-enzyme complex in the absence of glycine, but not in its presence.

The continued study of benzyl alcohol dehydrogenase would be most rewarding if several different mutant forms of the enzyme could be created by site-directed mutagenesis. Notably, the Val residue which replaces the normal His51 residue could be replaced by His, thus recreating the entire proton-
shuttle system known to exist in all the other members of
this alcohol dehydrogenase family. This approach will elimi-
nate all the problems associated with the use of external
proton acceptors to study the proton-shuttle system.

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