The Dual Effects of Alcohols on the Kinetic Properties of Guinea Pig Liver Cytosolic \( \beta \)-Glucosidase*

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This report demonstrates the effect of primary alcohols on the kinetic properties of guinea pig liver cytosolic \( \beta \)-glucosidase. Lineweaver-Burk analyses of the kinetic data revealed a biphasic response; at low concentrations the alcohols increased the \( V_{\text{max}} \) 5-7-fold while at higher concentrations they caused a purely competitive type of inhibition. For example, with n-butyl alcohol, increasing the alcohol's concentration in the assay medium from 0 to 0.14 M (0-1% (v/v)) resulted in a progressive increase in \( V_{\text{max}} \) to a value 7-fold above the basal level without affecting the \( K_m \). However, between 0.14 and 0.54 M (1 and 4% (v/v)) n-butyl alcohol, the \( K_m \) for 4-methylumbelliferyl-\( \beta \)-glucopyranoside increased significantly from 0.14 to 0.93 mM. In contrast to n-butyl alcohol or isobutyl alcohol, which are potent activators, structurally related compounds like sec-butyl alcohol, tert-butyl alcohol, butylurea, and butanesulfonic acid did not stimulate the activity of the cytosolic \( \beta \)-glucosidase. In the concentration range where activation was observed, conventional secondary replots of 1/\( \Delta \) slope versus 1/[alcohol] yielded perfect straight lines, demonstrating that binding of a single molecule of alcohol to the \( \beta \)-glucosidase was responsible for the initial phase of activation. Furthermore, the glycohydrolase displayed a propensity to bind the longer chain alcohols, as reflected by the \( K_d \) (binding constant) values of 5.55, 146, 34.1, and 7.47 mM for ethanol, n-propyl alcohol, n-butyl alcohol, 1-pentanol, respectively.

This phenomenon of nonessential activation by alcohols has led us to speculate on the presence of a physiologic activator for the \( \beta \)-glucosidase in mammalian tissues which contain this enzyme.

An outstanding feature of the prominent cytosolic \( \beta \)-glucosidase of mammalian liver is its broad specificity (Daniels et al., 1981). This broad specificity applies to both the glycone and aglycone moieties of the enzyme's substrates, for the glucosidase catalyzes the hydrolysis of a variety of nonphysiological glycosides of p-nitrophenol and 4-methylumbelliferone including \( \beta \)-D-glucosides, \( \beta \)-D-galactosides, \( \alpha \)-L-arabinosides, \( \beta \)-D-fucosides, and \( \beta \)-D-xylosides. Furthermore, this striking aspect of its catalytic flexibility is underscored by the recent finding that several plant aryl glucosides with widely differing aglycone moieties could serve as potential physiologic substrates (LaMarco and Glew, 1986). The latter observation has led to the suggestion that this relatively nonspecific glucosidase could play a role in the detoxification of xenobiotic glycosides which might find their way into the diet of man or animals. Since the highest specific activity is usually obtained with aryl \( \beta \)-D-glucoside substrates, this enzyme is referred to by many investigators as a \( \beta \)-glucosidase.

The broad specificity \( \beta \)-glucosidase has been purified to homogeneity from a variety of sources including human liver (Daniels et al., 1981) and guinea pig liver (LaMarco and Glew, 1986). The guinea pig liver \( \beta \)-glucosidase is fully active as a 60,000-dalton, single chain protein. Since it has maximum activity in the pH 5.7-6.5 range and in order to distinguish it from lysosomal glucocerebrosidase/\( \beta \)-glucosidase, which has a more acidic pH optimum, it is sometimes referred to as a “neutral” \( \beta \)-glucosidase.

Although its physiologic function remains to be established, we are interested in the structure of this soluble cytosolic \( \beta \)-glucosidase, particularly the hydrophobic domains located in its active site and elsewhere, because of several observations: first, its nominal (2-3-fold) activation by gangliosides such as Gn14; second, the mixed-type inhibition caused by micromolar concentrations of physiologically relevant amphiphilic compounds like glycosylphosphatidylinositol and galactosylphosphatidylinositol (LaMarco and Glew, 1985); and finally, its high affinity for hydrophobic affinity columns (e.g. phenyl- and octyl-Sepharose) (LaMarco and Glew, 1986).

In addition, in a recent comparative study (Gopalan et al., 1989) of the inhibitory effects of a homologous series of n-alkyl-\( \beta \)-D-glucosides, which act at the active sites of the human liver cytosolic \( \beta \)-glucosidase and placental lysosomal glucocerebrosidase, we observed that the inhibition constants (\( K_i \) values) for the former were at least 100-fold lower than those of glucocerebrosidase, which is an intrinsic membrane protein. This latter observation indicates that the active site of the broad specificity \( \beta \)-glucosidase may be as apolar or perhaps even more so than that of glucocerebrosidase which catalyzes the hydrolysis of the highly hydrophobic glucocerebroside.

In an effort to learn more about these hydrophobic binding sites on the cytosolic \( \beta \)-glucosidase, we investigated the effects of alcohols on the kinetic properties of the enzyme. In this report we show that the enzyme possesses two distinct binding sites for alcohols: a high affinity activator site and a low affinity site which is probably a part of the hydrophobic domain in the active site.

**EXPERIMENTAL PROCEDURES**

**Materials**

Cytosolic \( \beta \)-glucosidase was purified from the liver of the English short-hair (albino) guinea pig as described elsewhere (LaMarco and

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n-Butyl alcohol, isobutyl alcohol, ethanol, n-propyl alcohol, isopropyl alcohol, sodium citrate, ammonium hydroxide, sodium phosphate, sodium chloride, 2-mercaptoethanol, sodium azide, and ethylene glycol were purchased from Fisher. Sodium butyrate, butyraldehyde, butanesulfonic acid (sodium salt), butyramide, 1-pentanol, and 1,4-butanediol were purchased from Aldrich. sec-Butyl alcohol was purchased from Kodak. tert-Butyl alcohol was obtained from J. T. Baker Chemical Co. All solvents were distilled immediately before use.

Evaluation of Kinetic Data—Lineweaver-Burk analyses were performed in the presence of fixed alcohol concentrations using eight different MUGlc concentrations in the range 0.5-5.0 mM. The initial reaction rates and their corresponding substrate concentrations were subjected to a computer analysis which fits the data to a rectangular hyperbola using the mathematical procedure described by Cleland (1979) and one which places greater weight on values obtained at the higher substrate concentrations. The computer program calculates \( K_a, V_{max} \), and \( K/V_{max} \) values and the standard errors for these data. The means of the standard errors for \( K_a \) and \( V_{max} \) values in all our kinetic analyses averaged 10 and 4\%, respectively.

Binding constants (\( K_a \) values) for the alcohol-\( \beta \)-glucosidase interaction were calculated from secondary replots of \( 1/\Delta \) slope versus \( 1/\delta \) intercept versus \( 1/\text{[alcohol]} \) (Segel, 1975). The \( \Delta \) slope values were obtained from individual Lineweaver-Burk plots. The constants \( \alpha \) and \( \beta \) refer to the \( \Delta \) change in \( K_a \) and \( V_{max} \), respectively, obtained in the presence of a nonessential activator. In the \( 1/\Delta \) slope versus \( 1/\text{[alcohol]} \) plot, the \( x \) and \( y \) intercepts correspond to \( -\delta/\alpha K_a \) and \( -\delta/\beta V_{max} K_a/\beta (\beta - \alpha) \), respectively. By assigning values for \( \alpha \) and \( \beta \) it is possible to estimate accurately the \( K_a \) values.

RESULTS

The Effects of Various Alcohols and Related Substances on \( \beta \)-Glucosidase Activity—It has been suggested (Schier and Scheraga, 1962) that when one investigates the apolar interactions between a protein and an organic solvent the ambiguity in interpreting the biological response induced by changes in the dielectric constant and pH of the medium can be minimized by analyzing the effects at relatively low concentrations of individual members of a homologous series of organic compounds on the protein's structure or function. Hence, we undertook to examine the effect of a family of straight-chain alcohols on the kinetic properties of cytosolic \( \beta \)-glucosidase.

The standard \( \beta \)-glucosidase assay was used to investigate the effects of various concentrations of ethanol, n-propyl alcohol, n-butyl alcohol, and 1-pentanol on the specific activity of guinea pig liver \( \beta \)-glucosidase. From the results of this experiment which are presented in Fig. 1, one can draw at least three conclusions: 1) The alcohols brought about a biphasic activity versus concentration curve; at lower concentrations they stimulated \( \beta \)-glucosidase activity (5-7-fold) while at higher concentrations they began to inhibit activity. 2) The concentration of each alcohol at which maximum stimulation was achieved was lowest for 1-pentanol, the most apolar member of the series. 3) The order of their effectiveness as \( \beta \)-glucosidase activators was 1-pentanol > n-butyl alcohol > n-propyl alcohol > ethanol. The biphasic activity versus concentration curve observed with the alcohols has been observed in several other systems; it is noteworthy, however, that the concentrations of alcohols, especially 1-pentanol and n-butyl alcohol, used in our studies were much lower than those used by others (Tan and Lovrien, 1972 and references therein).

In order to gain insight regarding the specificity of the effects of these alcohols, we compared the ability of several other four-carbon compounds against n-butyl alcohol for their ability to stimulate the \( \beta \)-glucosidase. As shown in Fig. 2, whereas n-butyl alcohol activated the enzyme 7-fold, compounds in which the hydroxyl group of n-butyl alcohol was replaced by a carboxyl (butyric acid), sulfonyl (butanesulfonic acid), urea (butyurea), or amino (butylamine) group were ineffective as activators of the \( \beta \)-glucosidase. Thus, the hydroxy group of n-butyl alcohol appears to be critical for the \( \beta \)-glucosidase-stimulating effects of this alcohol. 1,4-Butanediol was only 30\% as effective as n-butyl alcohol in this regard, suggesting that the methyl group of n-butyl alcohol enhances binding of n-butyl alcohol to the enzyme.

We next compared the four isomers of butanol to determine if there might be structural requirements for the interaction.

FIG. 1. Effect of primary alcohols on \( \beta \)-glucosidase activity. Activity was determined using the standard \( \beta \)-glucosidase assay. Relative activity of \( \beta \)-glucosidase is plotted versus the concentration of ethanol ( ), n-propyl alcohol ( ), n-butyl alcohol ( ), or 1-pentanol ( ).

FIG. 2. Effect of n-butyl alcohol and related four-carbon compounds on \( \beta \)-glucosidase activity. The relative activity of \( \beta \)-glucosidase is plotted versus the concentration of n-butyl alcohol ( ), butyric acid ( ), butanediol ( ), butanesulfonic acid ( ), or butyurea ( ).
between the four-carbon alcohol and the cytosolic β-glucosidase. As shown in Fig. 3, other than n-butyl alcohol, only isobutyl alcohol approached n-butyl alcohol in terms of its ability to enhance β-glucosidase activity; the sec and tert isomers of butanol were essentially inactive in this regard.

Taken together, these data suggest that a relatively specific interaction occurs between these primary alcohols and the enzyme. Furthermore, they show that the length of the carbon chain, the shape of the molecule, and the presence of the hydroxyl group seem to be critical determinants of this interaction.

Lineweaver-Burk Analysis of the Effects of Primary Alcohols on Cytosolic β-Glucosidase—The interactions of the four normal alcohols ranging in carbon number from C₅ (ethanol) to C₅ (1-pentanol) with the β-glucosidase were analyzed kinetically. A typical Lineweaver-Burk analysis of the effects of n-butyl alcohol on the kinetic properties of the enzyme is shown in Fig. 4. It is apparent from the results shown in Panel A that at relatively low concentrations (0–0.14 M) the effect of n-butyl alcohol is exclusively to increase Vₘₐₓ without altering the Kₘ for MUGlc. Interestingly, as the concentration of n-butyl alcohol in the assay medium was increased from 0.14 to 0.54 M (Panel B), a very different pattern was observed; in this case, the slopes increased with increasing alcohol concentration and the Lineweaver-Burk plots intersected on theordinate, indicating purely competitive inhibition. Similar kinetic patterns were obtained when the effects of ethanol, n-propyl alcohol, and 1-pentanol on β-glucosidase activity were examined by Lineweaver-Burk analysis.

All of the kinetic data described above were expressed in terms of relative Vₘₐₓ/Kₘ ratios and plotted versus alcohol concentration; from the resultant patterns obtained, the bi-phasic nature of the alcohol effects on the kinetic properties of the enzyme are readily apparent (Fig. 5). For each of the four alcohols, at the lower concentrations, the replots exhibited a positive slope, indicating that at these concentrations the alcohols act as activators. Approximately the same normalized Vₘₐₓ/Kₘ ratio (5.0–7.0) is reached for all of the alcohols. Higher alcohol concentrations result in a negative interaction between the two alcohols and the enzyme. As shown in Fig. 3, other than n-butyl alcohol, only isobutyl alcohol approached n-butyl alcohol in terms of its ability to enhance β-glucosidase activity; the sec and tert isomers of butanol were essentially inactive in this regard.

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The present study demonstrates that amphipathic primary alcohols exert either a stimulatory or an inhibitory effect on the broad specificity cytosolic β-glucosidase of mammalian liver, depending on the alcohol concentration of the assay medium. At low concentrations the alcohols stimulate β-glucosidase activity, while at higher concentrations they inhibit its activity (Fig. 1). The enhancement of activity obtained with the lower concentrations of the n-alkyl alcohol series is entirely the result of a V_{max} effect, while the decrease in activity observed at the higher concentrations is completely accounted for by competitive inhibition (Fig. 4).

DISCUSSION

The results of the comparative study of the effects of the four members of the butanol family on the activity of the broad specificity β-glucosidase provide evidence against a general solvent effect on the enzyme’s conformation or on the assay medium (Fig. 3); instead, they favor a structurally specific interaction between the perturbant molecule (i.e. the alcohol) and the enzyme. Furthermore, the structural specificity of the enzyme-alcohol interaction is borne out by the observation that compounds like butyric acid, butylamine, and butyrylurea did not stimulate the activity of cytosolic β-glucosidase (Fig. 2).

A model which would explain the data and account for the interactions of the various amphipathic alcohols with the β-glucosidase consists of two elements: first, an anchoring component, which we attribute to the interaction of the hydrophobic domain of the alcohol with a hydrophobic site on the enzyme, and second, the proper orientation of the hydrophilic domain of the alcohol such that the hydroxyl group can interact with some polar group on the enzyme.

To explain the progression from activation to inhibition as the concentration of the alkyl alcohol in the assay medium is increased, it is helpful to conceive that the cytosolic β-glucosidase possesses two distinct apolar alkyl binding domains, with greatly differing affinities for nonpolar effectors. We designate the high affinity site as the activator site and envision the low affinity site to be an integral part of the enzyme’s active site. Using this model, we envision that at low concentrations, alcohols such as n-butyl alcohol bind to the high affinity site and convert the β-glucosidase from one conformation (State I) to another (State II) (Fig. 8); the latter form is catalytically more efficient than the alcohol-free form of the enzyme, as is evident from the 5–7-fold increase in the V_{max}. We propose that in this alternate conformation the essential functional groups at the active center which affect the actual catalytic process are transformed to assume a more favorable orientation. The transition from State I to State II entails removing or decreasing certain kinetic or thermodynamic restrictions inherently imposed on State I. An identical situation has been reported by Dimroth et al. (1970) for the biotin carboxylase component of the acetyl-CoA carboxylase of Escherichia coli. Their Lineweaver-Burk analysis revealed that 10% (v/v) ethanol increased the V_{max} 5–7-fold without causing a detectable change in the K_{m} values for ATP-Mg, HCO_{3}, or d-biotin.

We propose that in the present study the inhibition caused by the higher concentrations of alcohols, which is largely competitive in nature, is the result of a second alcohol molecule binding to the alkyl binding subsite at the active center of the β-glucosidase when it is in State II. These kinetic data support a model which envisions that the broad specificity β-glucosidase contains a second hydrophobic binding site, one that is distinct from the aglycone binding subsite at the enzyme’s catalytic center. Furthermore, this second hydrophobic site on the enzyme has a higher affinity for n-butyl alcohol than does the active site. Hence, at low concentrations the alcohols bind to this higher affinity (stimulatory) activator site whereas at higher concentrations they also bind to the lower affinity (inhibitory) site that corresponds to the active site, thus accounting for the biphasic kinetic pattern we observed. Of course, direct binding studies are needed to confirm the postulate of two alcohol molecules binding to the cytosolic β-glucosidase.

Since the β-glucosidase-catalyzed reaction proceeds rapidly in the absence of alcohol, the alcohol-induced increase in the reaction velocity causes us to classify this phenomenon as a case of nonessential activation (Segel, 1975). In the alcohol-induced β-glucosidase transition from State I to State II there
is no change in $K_a$, but a 5–7-fold increase in $V_{max}$. From our extensive Lineweaver-Burk analyses of the $\beta$-glucosidase reaction at different concentrations of each alcohol throughout the activation phase, we plotted the reciprocal of the change in slope ($1/\Delta$ slope) versus the reciprocal of the activator (alcohol) concentration. These perfectly linear replots (Fig. 6) obtained for all four alcohols provide convincing evidence for the binding of one molecule of alcohol to the activator site. Since the activator caused only an increase in $V_{max}$, replots of $1/\Delta$ intercept versus $1/[\text{alcohol}]$ are also linear (data not shown).

In the extensive kinetic analyses of $1/\Delta$ slope versus $1/[\text{alcohol}]$ (Fig. 6), the Y intercept $\beta V_{max}/K_a(\beta - \alpha)$ is the same for all alcohols. This supports the hypothesis that the alcohols are interacting with the same activator site on the glycohydrolase and that when they bind this site they all induce the same transition from State I to State II.

The $1/\Delta$ slope versus $1/[\text{alcohol}]$ replots for each alcohol yield the $K_a$ value for the respective enzyme-alcohol interaction. From Panel A of Fig. 7, it is evident that for each additional carbon atom, the $K_a$ value for the enzyme alcohol interaction decreases by a factor of 4.0–4.5. This 4-fold decrease in $K_a$ values translates into an additional 887 cal/mol ($3\Delta G^\circ(K_a)/CH_2$) in the free energy of binding ($AG^\circ(K_a)$) to the activator site on the cytosolic glycohydrolase (Fig. 7, Panel B).

The binding of alcohols to a hydrophobic site on the cytosolic $\beta$-glucosidase is a thermodynamically favorable process, due to entropic stabilization resulting from a decrease in what Schrier and Scheraga (1962) refer to as the organized “ice-like” structure of water molecules surrounding the nonpolar moiety of the alcohol and also, of those which had been in contact with the exposed hydrophobic side chains of this alcohol binding site. It is well established that the tendency of amphiphatic substances to partition from the aqueous bulk phase into the apolar interior of a protein increases with the hydrophobicity of their nonpolar moiety (Tanford, 1980). Hence, the greater thermodynamic potential of the longer chain alcohols, manifested as a larger entropic contribution to their free energy of binding to the activator site, may account for their higher affinity for this site relative to the lower alcohols (Table I). Therefore, it is not unusual to observe an inverse relationship between the chain length of the alcohols and the breakpoint in the biphasic $V_{max}/K_a$ versus alcohol concentration plot (Fig. 5).

The theory of hydrophobic bonding proposed by Nemethy and Scheraga (1962) predicts that upon increasing the hydrocarbon length of the organic material added to the assay medium there should be an increase in the magnitude of any observed effect on the biological properties of the protein. For instance, the lowering of the transition temperature of ribonuclease for a given concentration of alcohol was found to increase as the chain length of the straight chain alcohol was increased (Schrier et al., 1965). Alternatively, for a homologous series of apolar compounds, it may require lower concentrations of a higher analog and higher concentrations of a lower member to evoke the same quantitative response.

What might be the role of the high affinity alcohol binding site on the $\beta$-glucosidase? The term “ambiguous enzyme” is applied to those enzymes whose subcellular distribution (e.g. cytosolic versus membrane-bound) is dictated by the metabolic status of the cell (Wilson, 1980). It has been suggested (Wilson, 1980) that the reversible binding of “soluble” enzymes to particular subcellular membranes may achieve the dual objectives of activation and regulation of catalytic activity. The observation that short-chain primary alcohols can activate the cytosolic $\beta$-glucosidase in vitro 5–7-fold raises an interesting question: Do mammalian cells which produce this enzyme contain cofactors or regulatory substances which resemble these alcohols in their ability to influence the catalytic capacity, substrate specificity, and, in addition, affect the subcellular distribution of the cytosolic $\beta$-glucosidase? It is conceivable that such natural substances could alter the overall kinetics of a subcellular membrane-bound system of enzymes by juxtaposing the $\beta$-glucosidase with another sequential enzyme of a metabolic pathway.

Our speculation regarding the existence of a physiological activator for the cytosolic $\beta$-glucosidase is supported by precedents in the literature (Dimroth et al., 1970; Sanwal et al., 1966). In one such instance, the activation of phosphoenolpyruvate carboxylase by ethanol replaced the requirement for acetyl-CoA as the allosteric activator, thereby demonstrating that ethanol could elicit the same favorable conformational change in the enzyme as the in vivo allosteric effector (Dimroth et al., 1970). Hence, it seems reasonable to postulate that the alcohols mimic some in vivo effector of the cytosolic $\beta$-glucosidase.

It may be that the ability of the cytosolic $\beta$-glucosidase to hydrolyze its true physiologic substrate in vitro can be demonstrated only when the assay medium is supplemented with this natural activator. This idea should stimulate investigators to conduct a meticulous search for the putative activator (Schrier et al., 1970; Sanwal et al., 1966). Hence, it seems reasonable to postulate that the alcohols mimic some in vivo effector of the cytosolic $\beta$-glucosidase.

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REFERENCES