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INHIBITION OF GLYCEROL DEHYDROGENASE FROM *AEROBACTER AEROGENES* BY DIHYDROXYACETONE, HIGH IONIC STRENGTH, AND MONOVALENT CATIONS

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SUMMARY

1. The NAD^+-linked glycerol dehydrogenase (glycerol:NAD^+ oxidoreductase, EC 1.1.1.6) from *Aerobacter aerogenes* was studied with respect to affinity for several substrates and inhibition by several mechanisms.

2. The affinity of the enzyme was somewhat higher for 1,2-propanediol than for glycerol. At pH 9.5 the K_m for 1,2-propanediol was 6.1 mM and for glycerol the K_m was 13 mM.

3. The reverse reaction:

$$\text{Dihydroxyacetone} + \text{NADH} + H^+ \rightarrow \text{glycerol} + NAD^+$$

showed substrate inhibition at dihydroxyacetone concentrations above 0.4 mM. The K_m for dihydroxyacetone was 0.13 mM at pH 7.7

4. The enzyme was strongly inhibited by Li^+ and to a lesser extent by Na^+ at higher concentrations. The K_i for Li^+ was approximately 40 mM at pH 9.5. This inhibition seems to be of a coupling type and appears to be noncompetitive with K^+, which has been shown to be an activator for the enzyme.

5. The enzyme was inhibited by high ionic strength solutions.

INTRODUCTION

NAD^+-linked glycerol dehydrogenases (glycerol:NAD^+ oxidoreductase, EC 1.1.1.6) have been found in several organisms[1-3]. The most thoroughly studied of these enzymes has been the one from *Aerobacter aerogenes*[1,4-6]. BURTON AND KAPLAN[1] reported the oxidation of glycerol in cell-free extracts of *Aerobacter aerogenes*. The system required NAD^+, and the product of the reaction was dihydroxyacetone. The reaction proceeded with glycerol in the presence of hydroxylamine, which inhibits alcohol dehydrogenase (alcohol:NAD^+ oxidoreductase, EC 1.1.1.1), indicating that

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glycerol dehydrogenase was distinct from alcohol dehydrogenase. $NADP^+$ was inactive as a substitute for NAD^+.

The activity of glycerol dehydrogenase has been reported to be maximal or nearly so with glycerol, 1,2-propanediol, and 2,3-butanediol[5,6]. The pH optimum is about 9 (see ref. 5). Glycerol dehydrogenase is strongly inhibited by 8-quinolinol, α,α-dipyridyl, and Zn^{2+}. It is relatively insensitive to EDTA and to diethyldithiocarbamate[5].

LIN AND MAGASANIK[5] reported the activation of glycerol dehydrogenase by NH_4^+, K^+, and Rb^+, but no mention was made of inhibition by Na^+ or Li^+.

It was found during a glycerol assay with the enzyme that some component of the reaction mixture was inhibitory. Experiments to discover something of the nature of this inhibition led to the present research.

METHODS

Enzyme

Partially purified glycerol dehydrogenase was obtained as a dry powder from Sigma Chemical Company. The powder was suspended in glass-distilled water and dialyzed against distilled water at 5° for 12 h. Activity of the dialyzed enzyme decreased about 25% in 24 h.

Chemicals

NAD^+ and NADH were purchased from Sigma Chemical Company. Dihydroxyacetone was obtained from Calbiochem. 1,2-Propanediol was the product of Eastman Organic Chemicals. Glycerol was a Baker Analyzed Reagent. All other chemicals were reagent grade. All reagents were dissolved in glass-distilled water except NADH, which was dissolved in 0.1 M glycine, adjusted to pH 7.7 with KOH.

Assay

The enzymatic activity was determined by measuring the rate of NAD^+ reduction or of NADH oxidation at 340 mμ with a Beckman Model DK recording spectrophotometer, using quartz cuvettes with a 10-mm light path. The final volume of all reaction mixtures was 3 ml. The forward reaction using glycerol or 1,2-propanediol as substrates was carried out in 33 mM glycine adjusted to pH 9.5 with KOH unless otherwise noted. Initial NAD^+ concentration was 0.33 mM. The reverse reaction using dihydroxyacetone as substrate was carried out in 33 mM glycine adjusted to pH 7.7 with KOH. Initial NADH concentration was 0.33 mM. The reaction was initiated by the addition of enzyme. Reference cuvettes contained all components of the reaction mixture except enzyme. Reactions were run at room temperature (24°). Reaction rates are expressed as absorbance per min. All concentrations are final concentrations. One enzyme unit is defined as that causing an initial rate of reduction of 1.0 μmole of NAD^+ per min.

RESULTS AND DISCUSSION

Affinity for substrates

K_m values for glycerol and for 1,2-propanediol were calculated, using Line-

weaver–Burk plots, to be 13 mM and 6.1 mM, respectively, at pH 9.5 with 17 mM K^+. These results are in agreement with those of LIN AND MAGASANIK[5] and of BURTON[6]. The K_m value for dihydroxyacetone was 0.13 mM at pH 7.7 with 13 mM K^+. This value is ten-fold lower than that reported by BURTON[6], who did not mention conditions used in the determination. As shown in Fig. 1, a double reciprocal plot of reaction rates against dihydroxyacetone concentration gives a characteristic substrate inhibition curve with dihydroxyacetone concentrations above 0.4 mM being inhibitory. Three separate experiments gave similar results.

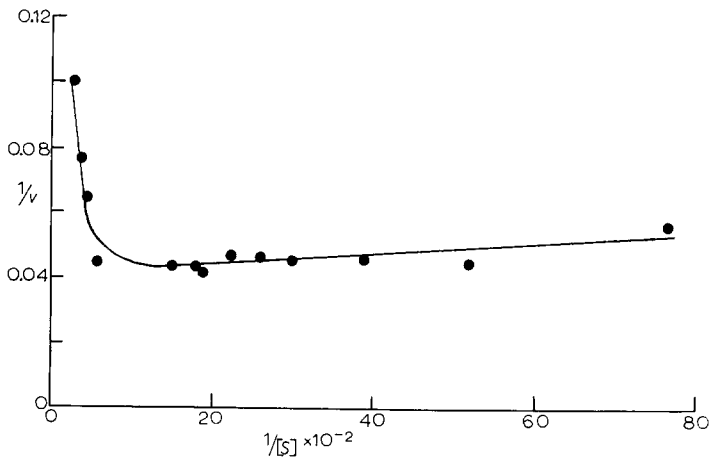

Fig. 1. Inhibition of glycerol dehydrogenase by dihydroxyacetone. The assay system consisted of 0.33 mM NADH, 33 mM glycine adjusted to pH 7.7 with KOH, and dihydroxyacetone. Final K^+ concentration was 13 mM. Reference cuvettes contained only buffer and substrate. Enzyme concentration was 0.02 units/ml.

Ionic strength and cation inhibition

LIN AND MAGASANIK[5] reported that the monovalent cations NH_4^+, K^+, and Rb^+ were activators of glycerol dehydrogenase, NH_4^+ being most effective and Rb^+ least effective. Addition of Na^+ or Li^+ to the reaction gave similar rates as those of the dialyzed enzyme in a reaction mixture containing no added cations. The reaction was run in $NaHCO_3$ buffer (pH 9.0), and final cation concentration was 17 mM. Preliminary results in our laboratory had suggested that Na^+ might be inhibitory, so a series of experiments were done to determine the effects of several cations. The results are summarized in Table I. Only 18% of maximum activity remained when the reaction was run in NaOH-adjusted buffer with no added K^+. Added NaCl at a final concentration of 670 mM did not significantly lower the reaction rate in the NaOH buffer. Addition of 670 mM K^+ to the NaOH-adjusted buffer restored activity to 62% of maximum. In a KOH-adjusted buffer there was a relative rate of 62% with 670 mM KCl but only 27% with 670 mM NaCl and 20% with LiCl added at the same concentration. Since K^+ is an activator for the enzyme, there was apparently an ionic strength effect to reduce enzyme activity. To test this possibility, the same salts were added to a KOH-buffer reaction mixture at final concentrations of 67 mM. As shown in Table I, activity was returned to maximum with K^+ while Na^+ and Li^+ were still inhibitory.

TABLE 1

IONIC STRENGTH AND CATION INHIBITION OF DIALYZED GLYCEROL DEHYDROGENASE

The assay system consisted of 20 mM glycerol, 0.33 mM NAD^+, and 33 mM glycine buffer adjusted to pH 9.5 with either KOH or NaOH as indicated. Final K^+ or Na^+ concentration was 15 mM when no salts were added to the mixture. Na^+, Li^+, or K^+ were added as the chlorides in the concentrations shown. Reference cuvettes contained all components of the experimental mixture except the enzyme. The enzyme concentration was 0.01 units/ml. Activities are given relative to the reaction rate in glycine–KOH buffer with no added salts. All concentrations are final concentrations.

Buffer	*Cation added*	*Concentration of added cation (mM)*	*Relative rate (%)*
Glycine–KOH	none	0	100
Glycine–NaOH	none	0	18
Glycine–NaOH	K^+	670	62
Glycine–NaOH	Na^+	670	18
Glycine–KOH	K^+	670	62
Glycine–KOH	Na^+	670	27
Glycine–KOH	Li^+	670	20
Glycine–KOH	K^+	67	107
Glycine–KOH	Li^+	67	33
Glycine–KOH	Na^+	67	76

Since Li^+ inhibition was apparently quite strong, further experiments were done to elucidate something of the nature of this inhibition. Reactions were run in 33 mM glycine–KOH buffer (pH 9.5) with 17 mM Li^+ and varied K^+ concentrations. The results are shown in Fig. 2 on a double reciprocal plot. It can be seen from this curve that the effect of Li^+ is to lower the K_m and to lower the V, *i.e.*, the affinity of the enzyme for glycerol is increased while the rate of product formation is decreased. These properties are characteristic of an uncommon type of inhibition called coupling or uncompetitive inhibition. Let us consider the following equations:

$$E + S \underset{}{\overset{K_S}{\leftrightharpoons}} ES \overset{k}{\rightarrow} E + P \qquad K_s = \frac{[E]\,[S]}{[ES]} \tag{1}$$

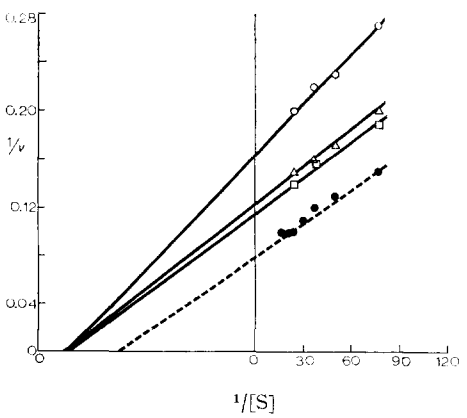

Fig. 2. Inhibition of glycerol dehydrogenase by Li^+. The assay system consisted of 33 mM glycine adjusted to pH 9.5 with KOH, 0.33 mM NAD^+, glycerol, and 0.02 units enzyme per ml. The dotted line represents the uninhibited reaction with 15 mM K^+. The inhibited reactions contained 17 mM Li^+ with 15 mM K^+ (○—○), 48 mM K^+ (△—△), and 65 mM K^+ (□--□). Similar results were obtained in three separate experiments.

$$E + I \overset{K_i}{\leftrightharpoons} EI \qquad K_i = \frac{[E]\,[I]}{[EI]} \tag{2}$$

$$EI + S \overset{\alpha K_s}{\leftrightharpoons} EIS \overset{\beta k}{\rightarrow} EI + P \qquad \alpha K_s = \frac{[EI]\,[S]}{[EIS]} \tag{3}$$

$$ES + I \overset{\alpha K_i}{\leftrightharpoons} EIS \overset{\beta k}{\rightarrow} EI + P \qquad \alpha K_i = \frac{[ES]\,[I]}{[EIS]} \tag{4}$$

where E is the enzyme, I is the inhibitor, S is the substrate, P is the product and where α represents the change in affinity and β the change in rate of complex breakdown induced by the inhibitor. Coupling inhibition occurs if $\alpha < 1$, *i.e.*, the inhibitor increases the affinity of the enzyme for the substrate, and $\beta < 1$, *i.e.*, the rate of product formation is decreased. The rate equation for coupling inhibition is

$$1/v = \left(1 + \frac{[I]}{K_i}\right)\frac{1}{V} + \left(\frac{K_s}{V}\right)\frac{1}{[S]} \qquad \text{(see ref. 10)} \tag{5}$$

References to coupling inhibition in the literature are few. Possible examples are the inhibition of cytochrome oxidase by azide[7] and of pepsin by hydrazine[8]. According to WEBB[9] the best documented examples are the inhibition of the arylsulphatase of *Alcaligenes metalcaligenes* by hydrazine and by cyanide[10]. These authors studied pH dependence of the inhibition and found a dissociating group in the enzyme–substrate complex which was not present in the enzyme alone. Furthermore, the inhibition increased sharply at a pH above the pK of this dissociating group, indicating that the inhibitors were reacting with this group. In addition, a family of parallel curves was obtained when $1/v$ was plotted against $1/[S]$ at different inhibitor concentrations.

In the present research, parallel lines were also obtained on a double reciprocal plot with different Li^+ concentrations (Fig. 3). The pH dependence of the inhibition was not studied in detail, but 17 mM Li^+ was shown to give 30% inhibition of the reverse reaction at pH 7.7 with 6.7 mM dihydroxyacetone and 13 mM K^+. Characteristic coupling inhibition curves were also obtained on plots of $[S]/v$ *vs.* $[S]$, v *vs.* $v/[S]$, $1/v$ *vs.* $[I]$, and on a Hunter-Downs plot. The co-ordinates of this type of plot are I ($[1 - i]/i$) *vs.* $[S]$, where $[I]$ is inhibitor concentration, i is fractional inhibition produced at given substrate and inhibitor concentrations, *i.e.*, 1 — (inhibited rate)/(uninhibited rate), and $[S]$ is substrate concentration.

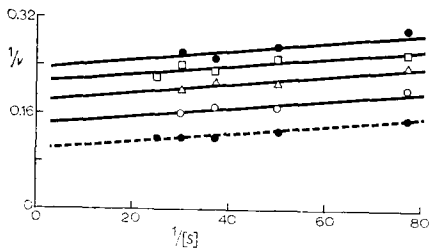

Fig. 3. Effect of different Li^+ concentrations on inhibition of glycerol dehydrogenase. The assay system consisted of 33 mM glycine adjusted to pH 9.5 with KOH, 0.33 mM NAD^+, glycerol, and 0.02 units enzyme per ml. Final K^+ concentration was 15 mM. The dotted line represents the uninhibited reaction. The inhibited reactions contained 26 mM Li^+ (●—●), 20 mM Li^+ (□—□), 13 mM Li^+ (△—△), and 6.7 mM Li^+ (○—○).

Preliminary experiments showed that additional K^+ could overcome Li^+ inhibition. Based on these data, it was supposed that a simple competition of Li^+ for the activator site could explain the inhibition. Fig. 2, however, demonstrates that simple competition for the activation site is not the explanation for Li^+ inhibition. It is clear from the double reciprocal plot that even though increasing K^+ concentration in the presence of Li^+ increased V, K_m was unaffected and remained at the decreased value caused by Li^+, *i.e.*, α remained constant while increasing the K^+ concentration increased β. What this means is that the product was formed more rapidly when the K^+ concentration was increased while the affinity of the enzyme for the substrate remained at the higher level induced by Li^+. Such a phenomenon would require that the Li^+ remain attached to the enzyme in the presence of increasing K^+ concentrations. Thus, Li^+ and K^+ must be bound at different sites on the enzyme. From the double reciprocal plot a K_i of 40 mM was calculated for Li^+.

Na^+ inhibition was more difficult to demonstrate. Concentrations of Na^+ high enough to be strongly inhibitory were near the concentrations causing ionic strength inhibition. However, it was possible to show on a double reciprocal plot inhibition kinetics similar to those for Li^+, though ten-fold greater concentrations of Na^+ were required to give as strong inhibition as given by Li^+.

The data presented do not suggest a mechanism of inhibition, but several aspects of the inhibition are noteworthy regarding a mechanism. Since the hydrated Li^+ is much larger than the hydrated K^+ and the hydrated Na^+ is intermediate, the size difference could explain the different effects of the three ions. In any case there appear to be separate sites for Li^+ inhibition and for K^+ activation. The fact that inhibition by Li^+ was obtained in the reverse reaction at pH 7.7 indicates that any ionizable group involved at the inhibition site has a pK lower than 7.7. A possible mechanism of inhibition that was not investigated is competition by Li^+ for the NAD^+ site, though this seems unlikely.

In view of the ionic strength inhibition, product inhibition, and the existence of several locations which affect enzyme activity in the presence of appropriate small molecules or ions, glycerol dehydrogenase seems to be an interesting enzyme for studying relationships of tertiary and quarternary structure and activity.

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