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ACTIVATION OF BOVINE LIVER GLYCEROL KINASE BY ETHANOL

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Summary

The crystallization of bovine glycerol kinase (ATP:glycerol 3-phosphotransferase EC 2.7.1.30) is reported along with a study of the unusual activation of this enzyme by ethanol. The enzyme was extracted from calf liver and purified 5900-fold giving crystals with a 5% yield. The kinetics of the enzyme with regard to glycerol and ATP were studied by varying the concentration of one substrate while keeping the other at saturating levels, and the effect of ethanol was observed by adding it at levels of 5% (v/v). Ethanol increased the V in both cases almost 2-fold. The apparent K_m of ATP was $3.5 \cdot 10^{-6}$ and was increased to $7.6 \cdot 10^{-6}$ in the presence of ethanol. The apparent K_m for glycerol was $3 \cdot 10^{-5}$ and was increased to $12 \cdot 10^{-5}$ when ethanol was added. A number of other alcohols had a similar activating effect except for 1,2-diols which only inhibited the enzyme. These findings are consistent with the explanation that alcohols compete with glycerol (hence also with the glycerophosphate product) for a hydroxy binding site on the enzyme. This leads to more rapid dissociation of the glycerophosphate (i.e. an increase in the steady-state constant, "k_{+2}" resulting in an increased V).

Introduction

In a study of the glycerol kinase of calf thyroid gland [1], we had noted that the enzyme was activated by ethanol. Since most enzymes are inactivated by ethanol, this behavior was considered unusual and further investigations into the effect of ethanol were undertaken. Since the thyroidal enzyme could not be crystallized we studied the calf liver glycerol kinase and this communication reports the crystallization of that enzyme and the effect of ethanol on its kinetics.

Methods

[2-^{3}H] Glycerol was obtained from New England Nuclear Corp. and diluted to 50 Ci/mol with unlabeled glycerol. The various alcohols used were reagent grade and obtained from standard commercial supply houses.

Assay

The details of the glycerol kinase assay have been previously described [1]. The assay is based on measurement of the conversion of radioactive glycerol into glycerophosphate. The standard assay system consists of 0.1 ml containing 0.1 M Tris · HCl buffer, pH 8.5 (at 37° C), 10 mM $MgCl_2$, 2 mM EDTA, 40 mM mercaptoethanol, 20 mM ATP, 1 mM [2-^{3}H]glycerol, 1 mg/ml of bovine serum albumin, and the sample to be assayed. For the given pH and temperature this composition of the assay gave the maximum reaction rate. After incubation for 10—20 min at 37° C the reaction is stopped by the addition of 0.1 ml *n*-propanol and the glycerophosphate formed is separated from the glycerol substrate by passing the reaction mixture over a small Dowex-1 (formate) ion-exchange column on which the glycerophosphate is adsorbed and eluted with CH_3OH/4 M HCl (4 : 1, v/v).

Results

Enzyme preparation and purification

The purification scheme is similar to the one used for calf thyroid glycerol kinase [1] and is based on previously reported techniques [2,3]. Except as noted, all operations were carried out at 4° C and the enzyme was kept in the presence of 1 mM EDTA, 1 mM mercaptoethanol, and 0.1 mM glycerol. Calf liver was transported from the slaughterhouse, on ice, to the laboratory where 1 kg was homogenized in a Waring blender with 4 l of cold 0.25 M sucrose. The homogenate adjusted to pH 5.1 with 2 M acetic acid and the sediment removed by centrifugation. Solid $(NH_4)_2SO_4$ was slowly added to the supernatant solution until 50% saturation was reached. After standing overnight the precipitate which formed was removed by centrifugation and dissolved in 375 ml of 0.01 M sodium acetate buffer (pH 5.1). This solution was quickly heated to 60° C in a steel beaker, held at that temperature for 20 min, cooled to 4° C, and centrifuged to remove the precipitate. The solution was brought to 30% saturation with solid $(NH_4)_2SO_4$, and after standing overnight the precipitate was removed. The $(NH_4)_2SO_4$ concentration was then raised to 40% of saturation and after 1 h the precipitate which formed was recovered and dissolved in a minimum of 0.01 M acetate buffer (pH 5.1). The preparation was applied to a 2.5 × 45 cm column of Sephadex G-150 which had been equilibrated with 0.02 M potassium phosphate buffer (pH 7.4) containing 1.0 mM glycerol. The column was eluted with the same phosphate buffer and the fractions of peak activity (92—136 ml) were pooled and applied directly to a 1 × 15 cm column of DEAE-cellulose (Whatman DE-23) equilibrated with the same phosphate buffer as above. This column was eluted with a gradient generated by the addition of 0.02 M phosphate buffer (pH 6.0) containing 0.5 M KCl to 0.02 M phosphate buffer (pH 7.4) maintaining a constant 300 ml in the mixing cham-

TABLE I

PURIFICATION OF BOVINE LIVER GLYCEROL KINASE

Units are defined as International Enzyme Units (1 unit = 1 μmol/min of product formed under conditions of our assay).

Fraction	Specific activity (munits/mg protein)	Relative purification	Yield* (%)
Homogenate	4.3	1	100
pH 5 supernatant	16	3.8	106
50% $(NH_4)_2SO_4$	50	11.6	96
Heat supernatant	163	38	71
30—40% $(NH_4)_2SO_4$	480	110	48
Sephadex fraction	670	155	37
DEAE fraction	3600	840	10
First crystallization	15000	3500	7.6
Second crystallization	25500	5900	5.2

* Total amount enzyme at start = 420 units/kg liver.

ber. Peak activity emerged between 120 and 200 ml and these fractions were pooled and brought to 70% saturation with $(NH_4)_2SO_4$ which caused the enzyme to precipitate overnight. The enzyme was dissolved in a minimum of 0.1 M potassium phosphate buffer (pH 6) containing 1 mM EDTA, 1 mM mercaptoethanol, and 0.1 mM glycerol and the solution was adjusted to 10% saturation with a solution of $(NH_4)_2SO_4$. After 2 days at 4°C a silky precipitate formed which was collected by centrifugation, dissolved in the phosphate buffer and recrystallized by slowly raising the $(NH_4)_2SO_4$ concentration to 20% saturation over 4 days. The crystals were recovered and suspended in a solution of the same composition. Table I lists the results at various steps in the purification of the enzyme. Under the microscope, the crystals were needle-like resembling those of pigeon liver glycerol kinase [4].

Disc gel electrophoresis on 6% acrylamide gel was carried out according to Hayashi and Lin [3]. A single protein band was observed which demonstrated enzyme activity with a specific stain for glycerol kinase [3].

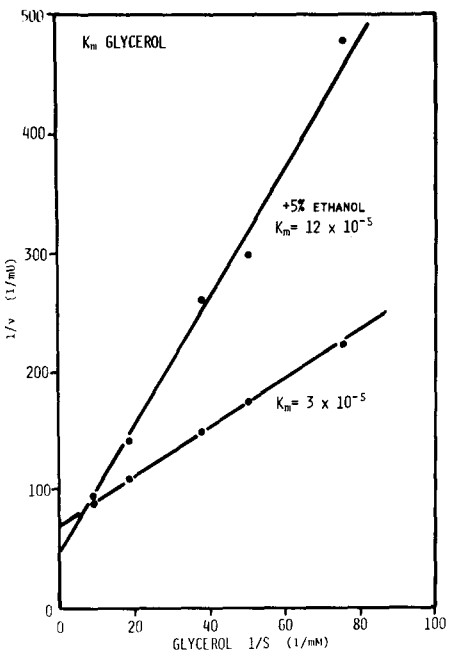
pH optimum

The response to pH of the calf liver glycerol kinase activity is similar to that demonstrated for the thyroidal enzyme [1]. Maximal activity occurs at a pH of 9—9.5 with a rapid fall off above 9.5 and below 6.5.

Kinetics and ethanol activation

The response of enzyme reaction rates to variations in the substrate concentration is plotted in Figs 1 and 2 according to the method of Lineweaver and Burk, both in the absence and presence of 5% (v/v) ethanol (0.87 M). When glycerol concentration was varied ATP was kept at 0.02 M, a level which is about 2000 times the apparent K_m of ATP in the presence of ethanol. The reactions were run for 10 min but were shown to be linear at least for 20 min.

For the determination of the K_m for ATP, the glycerol concentration was kept at 1 mM which was 10 times the apparent K_m of glycerol in ethanol. Under the conditions of a low ATP concentration the enzyme is relatively



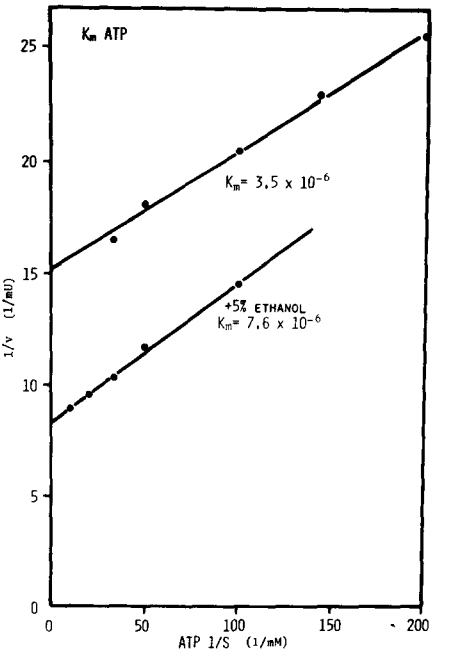


Fig. 1. K_m of glycerol and the effect of 5% (v/v) ethanol. Assays were run with a reaction time of 10 min in the standard assay system with 20 mM ATP and 10 mM $MgCl_2$. Each point is the mean of duplicate determinations. Velocity is expressed as apparent enzyme activity (1 munit = 1 nmol/min of product formed).

Fig. 2. K_m of ATP and the effect of 5% (v/v) ethanol. Assays were run with a reaction time of 5 min in the standard assay system ($MgCl_2$ and EDTA kept constant at 10 mM and 2 mM, respectively) with a glycerol concentration of 1 mM. Each point is the mean of triplicate determinations. Velocity is expressed as apparent enzyme activity (1 munit = 1 nmol/min of product formed).

unstable, especially in the presence of ethanol, so that shorter reaction times were necessary. Under the conditions used in Fig. 2, the reaction was found to be linear at least for the 5 min actually used for the assay, thus ensuring that the data represented initial velocities.

The $Mg \cdot ATP^{2-}$ complex is presumably the active substrate species but with the excess of Mg^{2+} at the pH of the assay essentially all the ATP is thus complexed and the concentration of the active species is equivalent to that of the ATP [5]. A variation of the $MgCl_2$ concentration by 50% in either direction did not affect the reaction rates.

The K_m for ATP was determined as $3.5 \cdot 10^{-6}$ increasing to $7.6 \cdot 10^{-6}$ in 5% ethanol and the K_m for glycerol was $3 \cdot 10^{-5}$ increasing to $12 \cdot 10^{-5}$ in ethanol. Ethanol also served to increase the V for each substrate.

Effect of various alcohols

To determine whether ethanol was unique, various alcohols were surveyed for their effect on glycerol kinase activity by adding them to the standard assay system at graded concentrations. Table II lists the results of these experiments giving the concentration at which maximum activity was observed and the

TABLE II

The effect of various alcohols on bovine glycerol kinase activity. Assays were performed in the standard assays system with a 20 min reaction time. The alcohols were added at graded concentrations beginning at a level below that causing maximal activation (group A) or below that causing any inhibition (group B). Concentrations were increased until significant inhibition was noted. Group A is composed of alcohols showing activation at some concentration while group B alcohols produced only inhibition without activation at any concentration.

	Maximum enhancement (percent of control)	Concentration (M)
Group A: Enhancing		
Methanol	230	5
Ethanol	220	2
1-Propanol	220	1
2-Propanol	170	2
1,3-Propanediol	190	1
1-Chloro-2-propanol	150	0.2
2-Chloro-ethanol	150	0.3
Ethanediol methyl ether	150	2
1-Butanol	230	0.5
2-Butanol	140	0.2
2-Methyl-1-propanol	220	0.2
2-Methyl-2-propanol	210	0.5
1,3-Butanediol	160	0.05
1,4-Butanediol	190	0.1
2,3-Butanediol	240	0.05
2-Pentanol	130	0.2
3-Methyl-1-butanol	130	0.2
Cyclohexanol	120	0.5
Group B: Inhibitory		
	Concentration for 50% inhibition (M)	
Ethanediol	0.2	
1,2-Propanediol	0.07	
3-Chloro-1,2-propanediol	0.2	
1-Pentanol	0.1	

percent activation at the concentration. Inhibition was eventually observed as the alcohol concentration was raised unless the concentration was limited by insolubility of the alcohol in the aqueous medium. Ethanol was tolerated at 15% (2.6 M) with an apparent enzyme activity of 165% of control but at 20% (3.4 M) the activity was depressed to 75% of control.

The reactions were run for 20 min but no attempt was made to determine whether initial velocities were maintained for that interval. Rather, the survey provides a rough grouping of the tested compounds into those that at some concentration enhanced glycerol kinase activity and others which did not stimulate activity but only inhibited it as their concentrations were raised.

Effect of ethanol on other glycerol kinases

Ethanol added to the crystallized glycerol kinases of *C. mycoderma* (Boehringer) and *Escherichia coli* (gift of Dr E. Lin) in graded concentrations (beginning with levels that had no effect) caused only inhibition of activity.

Similarly, ethanol caused only inhibition of the glycerol kinase activity of guinea pig, rabbit, and rat liver homogenates. The activity of dog and human liver homogenates showed at most a 10% increase in activity in the presence of

ethanol. For comparison calf liver homogenate showed a maximal enhancement to 200% of control in 10% (1.7 M) ethanol.

Discussion

Since ethanol inactivates most enzymes at room temperature it is unusual to find an enzyme that even at 37° C is apparently activated by ethanol. This property appears even more unique since only the bovine glycerol kinase and perhaps, marginally, the canine and human enzymes, are activated while the glycerol kinases of *C. mycoderma*, *E. coli*, rat, guinea pig, and rabbit are not.

The kinetics of the enzyme in the presence of ethanol also appear unusual in that the alcohol increases maximum velocity (V) as well as the Michaelis constant (K_m) for both ATP and glycerol substrates. Ethanol thus acts as an inhibitor when substrate concentrations are low but as an activator when they are high. Similar kinetics were observed by Theorell and McKinley-McKee [6] in the case of liver alcohol dehydrogenase exposed to imidazole. They concluded that the effect of imidazole was to destabilize the ternary complex of enzyme and reaction products (aldehyde and NADH) leading to a more rapid release of the products. Our kinetics are most easily explained by the same mechanism. One can postulate that the hydroxy group of the ethanol (and of the other alcohols) competes with only one of the hydroxy binding sites of glycerol (allowing some binding at a second hydroxy site) with relatively little effect at the reactive phosphorylation site. This would decrease the binding not only of the glycerol substrate but also of the glycerolphosphate product and, in effect, destabilize the ternary complex. This can also be viewed as an increase in "k_{+2}" (the leaving rate of the rate limiting product) in the Briggs-Haldane expression:

$$K_m = \frac{k_{-1} + k_{+2}}{k_{+1}} \quad , \quad V = k_{+2} \cdot e.$$

Since the Lineweaver-Burk plot for each substrate under our conditions was linear, it is reasonable to suppose that this expression holds for each substrate while the other is saturating. Since we are considering initial reaction rates the rate-limiting step and, hence, k_{+2} is the same regardless of which substrate is taken as saturating. Ethanol, by interfering with glycerol binding, would also increase k_{-1} and decrease k_{+1} and thus contribute to an increase in the K_m of glycerol. The increased K_m for ATP can similarly be explained by an increase in k_{+2} with a possible contribution of ethanol effects on the k_{-1} and k_{+1} of ATP. In either case the increased V can only be explained by an increase in k_{+2} or by an increase in "e". An increase in e would represent an augmentation of active enzyme sites, induced by ethanol, but this seems less likely than an effect of ethanol at binding sites.

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