BRIEF COMMUNICATION

THE EFFECTS OF METHANOL ON THE GLUTAMATE DEHYDROGENASE REACTION AT 0°C

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ABSTRACT The effects of 0–30% methanol (vol/vol) on the K_m and V_m values for both the forward and reverse directions of the L-glutamate dehydrogenase reaction were determined at 0°C. The decrease in temperature alone to 0°C had very little effect on these parameters. However, in the forward reaction, 30% methanol resulted in a 14-fold decrease in the K_m value for glutamate, a slight decrease in the K_m value for NADP, and a thirty-fold decrease in V_m. Substrate inhibition by glutamate was observed at concentrations greater than 4 mM. In the reverse reaction, 30% methanol caused a decrease in the K_m values for α-ketoglutarate and ammonia and a 10-fold decrease in V_m. Substrate inhibition by both α-ketoglutarate and NADPH was observed at concentrations of either substrate above 0.03 mM. The dependence of K_m for glutamate and V_m values for the forward reaction on methanol concentration suggests that they are similarly affected by methanol, in direct contrast to results obtained for NADP. Methanol appeared to cause a general tightening of complexes, which may arise from an effect on the "activities" of species in solution. The use of methanol not only allows for the study of reaction intermediates by slowing the reaction with the cryogenic method, but may also serve as a mechanistic probe by affecting solvent polarity as well as K_m, V_m, and K_i values.

INTRODUCTION

An often encountered problem in the study of enzyme reaction mechanisms is the inability to isolate and identify short-lived intermediates. Rapid reaction techniques, such as stopped-flow and temperature-jump spectrophotometry, have been used for this purpose, with some success. Recently, Fink et al. (1–4), Douzou et al. (5–7), Alber et al. (8), and Makinen et al. (9) have shown that at subzero temperatures, enzyme catalyzed reaction rates can be slowed sufficiently so that the catalytic reaction appears as a series of successive steps with one intermediate converted into the next as the temperature is raised until a high enough temperature is reached for turnover to occur.

One of the primary requirements of this method is the need for a solvent system that will remain fluid at subzero temperatures. Since the addition of an organic solvent to the reaction

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mixture has the potential of altering protein structure, ligand binding, catalytic activity, and water structure, it is necessary to determine the effects of the cosolvent itself on the kinetic parameters of the reaction, as well as the dependence of those effects on cosolvent concentration (1, 4, 7, 10, 11). In the course of such a preliminary study of the suitability of methanol as a cosolvent for cryogenic studies of the glutamate dehydrogenase-catalyzed reaction, we found a number of interesting effects that in themselves reveal some information about the nature of substrate-enzyme interactions. These effects on the steady-state kinetics are reported here.

MATERIALS AND METHODS

Materials

Beef liver L-glutamate dehydrogenase was purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.) as a crystalline suspension in 2 M ammonium sulphate, pH 7. Before use, the enzyme was exhaustively dialyzed against 0.1 M potassium phosphate buffer, pH 7.6, treated with approximately 1 mg/ml Norit A for 30 min, and filtered through a 0.45-μ Millipore filter (Millipore Corp., Bedford, Mass.). Both NADP (sodium salt form, Sigma grade) and NADPH (tetrasodium salt type III) were obtained from Sigma Chemical Corp. (St. Louis, Mo.). The NADPH was enzymatically reduced. L-glutamic acid and α-ketoglutarate were purchased from Calbiochem (Calbiochem-Behring Corp., San Diego, Calif.) and both were A Grade. Spectrophotometric grade methanol was obtained from Mallinckrodt Inc. (St. Louis, Mo.), as was analytical grade potassium phosphate dibasic. Ammonium acetate (ACS Certified) and potassium phosphate monobasic (Fisher Certified) were obtained from Fisher Scientific Co. (Pittsburgh, Pa.).

Methods

Enzyme activity was monitored by following the change in absorbance at 340 nm on a Gilford 2000 spectrophotometer equipped with a thermoprogrammer and specially designed rapid sampling thermocuvette (Gilford Instrument Laboratories Inc., Oberlin, Ohio). These attachments provided both for temperature control within $\pm$0.4°C, between 0°C and 100°C, and the rapid running of steady-state kinetics. Oxidative deamination of L-glutamate (the forward reaction) was run at 0°C and 25°C with 0.1 M potassium phosphate buffer, pH 8.6; L-glutamate varied from 0.2 to 5 mM; NADP varied from 0.2 to 1 mM; and enzyme varied from 0.001 to 0.2 mg/ml (0.02–4 μM). At 0°C, reactions were also run in the presence of 5, 15, and 30% methanol (vol/vol). In all cases, NADP was added to start the reaction. For the reverse reaction, fixed concentrations of 2 mM α-ketoglutarate, 0.1 mM NADPH, and 10 mM ammonium ion were used. Whenever any one of these became the varied substrate, concentrations of 0.025–1 mM for α-ketoglutarate, 0.02–1 mM for NADPH, and 1–7 mM for ammonium ion were used. L-glutamate dehydrogenase in the concentration range of 0.002–0.02 mg/ml (0.04–0.4 μM) was added to start the reaction.

The reverse reaction was run at both 0°C and 20°C, and pH was maintained at 7.6 by using 0.1 M potassium phosphate buffer. At 0°C, the reaction mixture was prepared both with and without 30% methanol. All Michaelis constants and maximum velocities for both forward and reverse reactions were determined from Lineweaver-Burk plots.

The ternary complex, L-glutamate dehydrogenase-reduced coenzyme-α-ketoglutarate, was prepared in 30% methanol at 0°C by using 2 mg/ml enzyme, 0.07 mM NADPH, and 0.5 mM α-ketoglutarate. Glutamate, 3.3 mM or 33 mM, was added to performed enzyme-reduced coenzyme-α-ketoglutarate (ERK) to form enzyme-reduced coenzyme-glutamate (ERG). The reaction was followed by observing λ_{max} of the peak in the 300–400 nm region versus time in a Cary 14 spectrophotometer (Cary Instruments, Monrovia, Calif.).

To detect any irreversible effects of methanol on catalytic activity, the enzyme was incubated in 30% methanol for either 5 min or 5 h at 0°C. Aliquots were removed and assayed under standard condi-

tions—33 mM glutamate, 0.5 mM NADP, and 0.005 mg/ml GDH at 20°C. The final concentration of methanol in the assay mixture was 0.4%.

pH measurements were taken on a Radiometer (type TTT1C) pH meter equipped with a radiometer scale expander, Type pHA630Ta, with a Radiometer combined glass electrode, type GK 2302C. Standardization buffers and assay solutions were cooled to 0°C for pH measurements (Radiometer, London Co., Westlake, Ohio). The pH of aqueous-organic solutions was determined by extrapolation from data published by Hui Bon Hoa et al. (12). Protein concentration was measured on a Zeiss M4 QIII spectrophotometer (Carl Zeiss Inc., NewYork) and calculated by using an extinction coefficient of 0.97 (13).

RESULTS AND DISCUSSION

Effects of Temperature

The effect of decreasing temperature alone on the K_m and V_m values of the forward and reverse reactions is shown in Table I. For oxidative deamination (henceforth referred to as the forward reaction), a decrease in temperature from 25 to 0°C causes the K_m for glutamate to increase twofold, the K_m for NADP to increase threefold, and the V_m to decrease approximately 15%. For the reverse reaction, the K_m's for the three reactants remain essentially unchanged when the temperature is dropped from 20 to 0°C, while the V_m decreases approximately threefold. The observed decrease in V_m for both the forward and reverse reactions is as would be expected from an overall decrease in the kinetic energy of the system. The K_m's, however, either increase or remain the same. Since the steady-state rate in the forward reaction is determined by the rate of dissociation of NADPH from tight ternary product complexes, elevation of the K_m would indicate that steady-state concentrations of these complexes must drop somewhat with temperature, possibly because the rate of formation is lower at lower temperatures. In the case of the reverse reaction, this effect is not observed.

Effects of Methanol

Methanol (up to 30% (vol/vol)) has no effect on the pH optimum of the reaction. Reaction rates in 30% methanol at 0°C for the forward reaction are optimum at pH 8.6. The same results are noted in aqueous solution at 0°C.

TABLE I

EFFECT OF TEMPERATURE ON K_m AND V_m*

	20°C, aqueous	25°C, aqueous	0°C, aqueous
Forward reaction			
K_m(glu)	—	0.31 mM	0.68 mM
K_m(NADP)	—	0.04 mM	0.11 mM
V_m‡	—	4.3	3.74
Reverse reaction			
K_m(α-KG)	0.11 mM	—	0.08 mM
K_m(NADPH)	0.016 mM	—	0.017 mM
K_m(NH_4^+)	22.2 mM	—	20 mM
V_m‡	14	—	4.3

*Assay conditions are described under methods.

‡Units for V_m are ΔA per minute per milligram protein.

TABLE II
THE EFFECT OF METHANOL ON K_m AND V_m*

	0°C, aqueous	0°C, 5% methanol	0°C, 15% methanol	0°C, 30% methanol
Forward reaction				
K_m (glu)	0.68 mM	0.34 mM	0.21 mM	0.05 mM
K_m (NADP)	0.11 mM	0.10 mM	0.085 mM	0.07 mM
V_m‡	3.74	2.07	1.25	0.11
Reverse reaction				
K_m (α-KG)	0.08 mM	—	—	0.038 mM
K_m (NH_4^+)	20 mM	—	—	4 mM
K_m (NADPH)	0.017 mM	—	—	N.D.§
V_m‡	4.3	—	—	0.45

*Assay conditions are described under Methods.
‡Units for V_m are ΔA per minute per milligram protein.
§K_m values for NADPH cannot be adequately determined under these conditions because the concentration of NADPH cannot be varied sufficiently. In the presence of methanol, substrate inhibition by NADPH occurs at concentrations greater than 0.03 mM; therefore, it is necessary to assay at very low concentrations of coenzyme. The change in optical density at 340 nm, however, is then not sufficient to obtain reliable rate measurements.

Enzymatic activity, recovered after incubation in 30% methanol, is indistinguishable from that of native enzyme, indicating that there are no significant irreversible effects of methanol on enzyme structure and reactivity.

Previous studies by Fink et al. (1, 4) on the effects of organic cosolvents on enzyme kinetics have shown K_m to increase with increasing concentration of organic cosolvent. More recently, studies by Ishida et al. (14) on carbamoyl phosphate synthetase have shown a decrease in K_m when either dimethylsulphoxide (DMSO) or glycerol are used as the cosolvent. Our studies with L-glutamate dehydrogenase present another case for an apparent decrease in K_m in the presence of an organic cosolvent, methanol. These results are shown in Table II. Increasing methanol concentration causes a significant decrease in K_m for glutamate in the forward reaction, and for ketoglutarate and ammonia in the reverse reaction. Methanol also causes a decrease in V_m for both the forward and reverse reactions. As methanol concentration increased from 0 to 30%, V_m for the forward reaction decreased approximately 30-fold, and for the reverse reaction, 10-fold. In contrast to previous results obtained by Fink (1, 4) on other systems, these decreases in kinetic parameters, in particular the decrease in reaction rate, is not simply in proportion to the decreased water concentration.

Table III shows an additional effect of methanol on the enzymatic reaction—the concentration of substrate needed to produce inhibition is significantly lowered. Substrate in-

TABLE III
THE EFFECT OF METHANOL ON SUBSTRATE INHIBITION

Substrate inhibitor	Concentration above which inhibition occurs	
	0°C, aqueous	0°C, 30% methanol
Glutamate	33 mM	4 mM
α-Ketoglutarate	1 mM	0.03 mM
NADPH	0.1 mM	0.03 mM

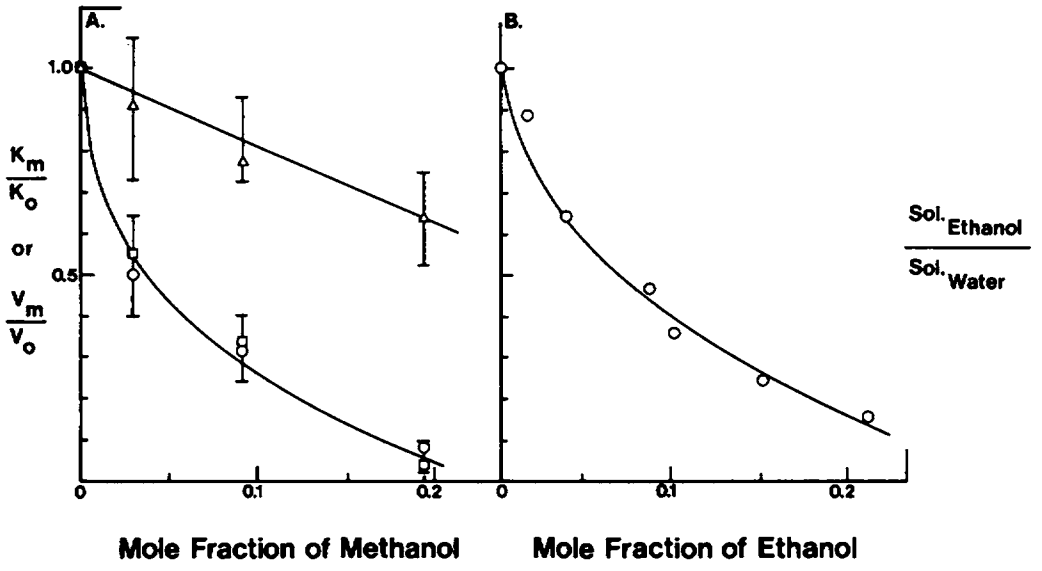
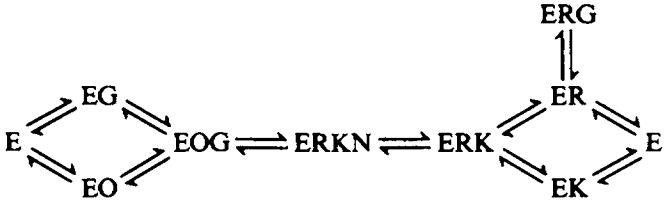


FIGURE 1 (A) The effect of increasing methanol concentration on the K_m values for glutamate (○—○), and NADP (△—△), and the V_m for the forward reaction (□—□); (B) the effect of increasing ethanol concentration on the solubility of L-aspartic acid (15). V_0 and K_0 are the V_m in aqueous solution and the K_m in aqueous solution, respectively.

hibition by glutamate in the forward reaction and either ketoglutarate or NADPH in the reverse reaction is observed at much lower concentrations of inhibitor when 30% methanol is added to the system at 0°C. The lower end of the concentration range required for inhibition by glutamate decreases from 33 mM (observed at 0°C in aqueous solution) to 4 mM. For ketoglutarate, the decrease is from 1 to 0.03 mM and for NADPH from 0.1 to 0.03 mM.

The methanol concentration dependence of the K_m values for glutamate and NADP and the V_m for the forward reaction is shown in Fig. 1. The comparable decreases both in the glutamate K_m and in the V_m observed at lower concentrations of methanol are in direct contrast to the results obtained for NADP.

The known reaction pathway including only the complexes formed under initial rate steady-state conditions for the glutamate dehydrogenase reaction is shown here:



where E is L-glutamate dehydrogenase; G, L-glutamate; O, NADP; R, NADPH; N, ammonia; and K, α-ketoglutarate. The observed effects of methanol on K_m's, V_m's, and substrate inhibition could be accounted for by a tightening of ternary complexes containing E and R.

The appearance of substrate inhibition at lower concentrations of glutamate would suggest a tightening of the ERG complex. However, model studies, using performed ERK in 30% methanol at 0°C with G added in excess, indicated that G did not displace K to form ERG. While formation of a tight ERK(N) would explain individual effects on the steady-state parameters, these interpretations cannot be justified by preliminary transient data in 30% methanol at 5°C. This would suggest that the situation is much more complex and that it cannot be explained in terms of the above simple mechanistic scheme.

The shapes of the curves shown in Fig. 1 suggest that methanol exerts a similar effect on the K_m for glutamate and the V_m, while it has a very different effect on the K_m for NADP. One explanation is that this may be due to an increase in hydrogen bonding or electrostatic interactions, arising from the effect of organic cosolvent on the "activities" of species in solution. The hydrophobic nature of the methanol cosolvent itself would favor formation of tight complexes over that of their more polar constituents. Hydrogen bonding or electrostatic interactions may also manifest themselves by a change in solubilities of the reactants. For example, amino acids such as glutamic acid are generally less soluble in methanol than in water. This effect alone would lead to an apparently tighter binding. Indeed, there is a resemblance between the curve shown in Fig. 1 for the effect of increasing methanol concentration on the K_m for glutamate, and the solubility curve obtained for an amino acid of similar structure, aspartic acid, in a similar organic solvent, ethanol. No information is available, however, about the effect of increasing methanol concentration on the activities of coenzyme or the functional groups at the enzyme active site.

Indeed then, not only have we demonstrated that we can study L-glutamate dehydrogenase using methanol as an antifreeze for cryogenic work, but we have learned that organic cosolvents may themselves prove to be effective mechanistic probes in the GDH system by allowing us to alter K_m, V_m, and K_I values, as well as affording us the opportunity of characterizing the relative polarity of transient reactive complexes.

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