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Study of fumarase activity in non-conventional media. Part II

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Abstract

The hydration of fumarate and the dehydration of L-malate catalysed by fumarase were investigated in water/methanol and water/formamide one phase systems. The effects of the amount of organic solvent on the maximum velocity (V_{max}), the Michaelis–Menten constant (K_M) and the equilibrium constant (K_{eq}) were studied for both the reaction media. The denaturing power of both methanol and formamide was observed together with the familiar decrease of the K_M. Fumarase catalysis in water/methanol systems was further investigated by evaporating the organic solvent and evaluating the degree of reversibility of the inactivation. Reversibility of formamide denaturation was also investigated. The effects of phosphate concentration in the reaction medium with different amounts of methanol was investigated following the variation of the kinetic parameters of the hydration reaction. At high concentrations of phosphate an inhibiting effect appeared. Time-dependent denaturation was also investigated and a remarkable instability of fumarase in systems with percentages (v/v) of formamide higher than 10% was observed. 10% formamide proved to be less deactivating than the other non-conventional reaction media so far employed.

Keywords: Fumarase; Organic solvents; Activity

1. Introduction

The effects of some organic solvents on fumarase activity, previously reported [1], showed that this enzyme is quite sensitive to the presence of even small amounts of organic solvent in the reaction medium; in particular a deactivating effect of ethylene glycol, glycerol and DMF was observed as their percentage in the medium was increased. Nevertheless it was found that these variations in the composition of the reaction medium deeply alter the enzyme's affinity for its natural substrates, fumarate and L-malate [2–4], causing a decrease of the K_M that evidenced an increase of such an affinity.

In order to test the effects of a broader range of organic solvents on enzyme activity, fumarase catalysis is now studied in water/methanol and water/formamide one phase systems. These organic solvents possess unique features that give a better understanding of the nature of the interactions between fumarase and the solvent itself: methanol has a higher vapour pressure than water and thus it was possible to eliminate it by evaporation from the reaction medium, while formamide has a dielectric constant much higher than water. These aspects offered the opportunity to investigate the reversibility of solvent denaturation and to study more fully the effect of solvent polarity on enzyme activity.

Furthermore, fumarase catalysis in

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water/methanol was carried out also for the dehydration reaction of L-malic acid permitting us to evaluate enzyme behaviour in the direct and reverse reactions.

2. Materials and methods

Fumaric acid disodium salt anhydrous puriss. > 99% was from Fluka Chemie (Buchs, Switzerland). L-Malic acid disodium salt monohydrate 98% was from Aldrich Chemical Co. (Milwaukee, WI, USA). Pig heart fumarase, as a crystalline suspension in $(NH_4)_2SO_4$ 3.2 M, KH_2PO_4 50 mM, 2-mercaptoethanol 14 mM pH 7.5, was from Sigma Chemical Co. (St. Louis, MO, USA). Its declared activity was 400 U/mg of protein and was experimentally confirmed. Methanol was from Carlo Erba (Milano, Italy) and formamide was from Aldrich Chemical Co.

Experimental details about reaction kinetics and reaction media are the same as described in the previous communication.

The evaporation of methanol from the reaction medium was carried out with a Speedivac Fs 100 oil pump for about 40 min. During the endothermic evaporation process the temperature remained at about 10°C thus avoiding thermal denaturation; once the evaporation finished the solution was diluted with phosphate buffer 50 mM pH 7.3 up to the original enzyme concentration. The content of residual methanol was determined gas chromatographically and was found to be less than 1% (m/v).

Dilutions of formamide solutions were done immediately after the preparation using phosphate buffer 50 mM pH 7.3 and enzyme concentration before dilution was such to give, after dilution, an enzyme concentration of 7.2 U/ml.

3. Results and discussion

Fumarase catalysis in aqueous system was firstly investigated for comparison and the results were shown in a previous communication [1,5,6].

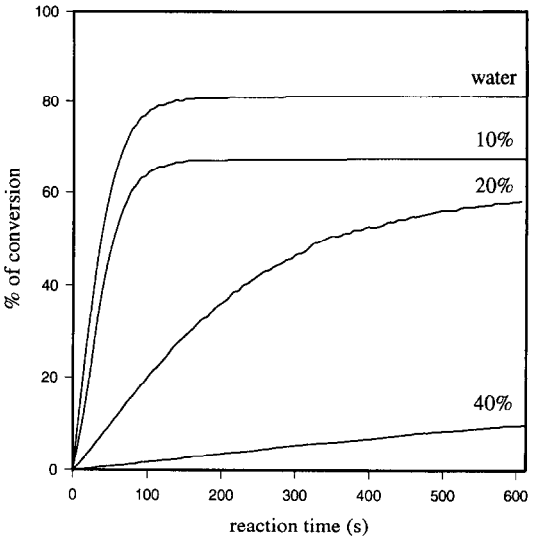

Fig. 1. Conversion vs. time of the hydration reaction of fumarate at different percentages of methanol in the reaction medium.

The biocatalysed hydration reaction of fumarate in water/methanol was studied using 8 different initial concentrations of substrate with a fumarase concentration of 7.2 U/ml. The percentage of conversion vs. time is reported in Fig. 1 showing the effect of the amount of methanol in the reaction medium, while in Fig.

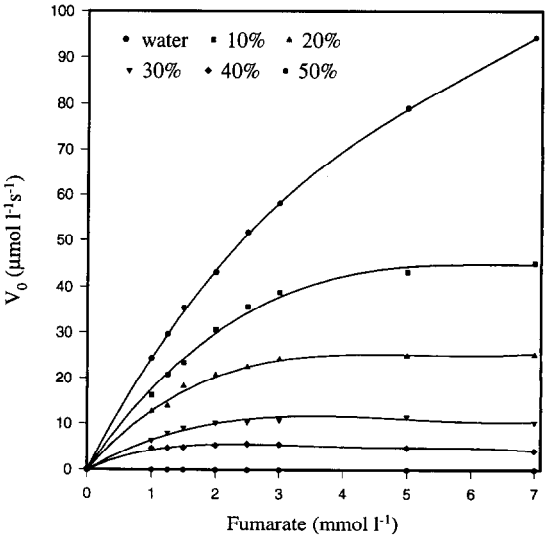

Fig. 2. Initial rate vs. fumarate concentration at different percentages of methanol in the reaction medium.

Table 1
V_{max}, K_M and K_{eq} values for the hydration reaction of fumarate in water/methanol as a function of methanol (v/v) percentage in the reaction medium

	water/methanol, as % methanol				
	3	10	20	30	40
V_{max} (μmoll^{-1} s^{-1})		82.7	38.2	15.4	6.90
K_M (mM)		3.84	1.95	1.26	0.56
K_{eq}	4.36	2.34	1.96	1.15	0.7

Conditions: fumarase = 7.2 U/ml, T = 25°C.

2 the initial rates vs. substrate concentration are plotted together with the values obtained in pure water.

V_{max}, K_M, and K_{eq} are listed in Table 1 as a function of methanol percentage in the reaction medium.

In 20% methanol the dehydration of L-malic acid was also studied in order to compare the effect of the organic solvent on both the direct and the reverse reactions catalysed by fumarase. The concentrations of L-malic acid used for the initial rate measurements were equal to the fumarate concentrations used for the study of the hydration reaction (the enzyme concentration was also the same).

The values of the initial rates of the dehydration reaction of L-malic acid in 20% methanol as a function of substrate concentration are reported in Table 2. The V_{max} and K_M values were 38.2 μmol l^{-1} s^{-1} and 4.71 mM, respectively. The percentages of conversion vs. time

Table 2
Initial rates of the dehydration reaction of L-malic acid in methanol 20% at different concentrations of substrate

S_0 (mmol l^{-1})	V_0 (μmol l^{-1} s^{-1})
1.00	6.7
1.25	8.3
1.50	8.9
2.00	11.1
2.50	13.3
3.00	15.5
5.00	20.0
7.00	22.2

K_M was 4.71 mM and V_{max} was 38.2 μmol l^{-1} s^{-1}. Conditions: fumarase = 7.2 U/ml, T = 25°C

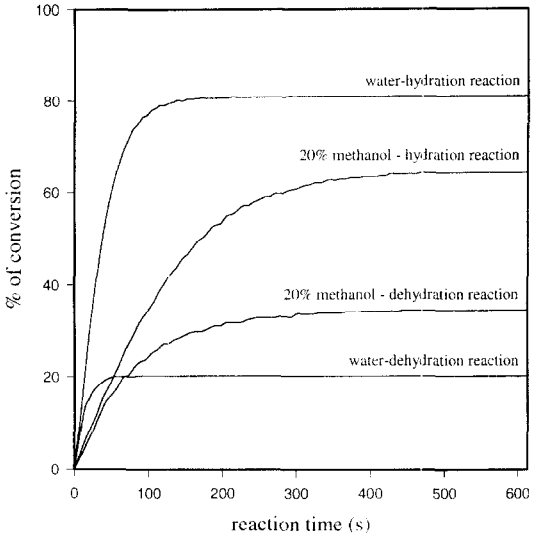

Fig. 3. Conversion vs. time of the hydration reaction of fumarate and the dehydration reaction of L-malate in water and in methanol 20%.

in 20% methanol and in pure water for both the hydration and dehydration reactions are shown in Fig. 3. A decrease of both V_{max} and K_M was observed also in the dehydration of L-malic acid showing a similar effect of methanol on the two directions of reaction: in 20% methanol the K_M drops to less than 1/3 the values in pure aqueous solutions. It is also possible that the differences of polarity between fumarate and L-malate may originate different kinetic behaviours when either the former or the latter are used as substrate, so that the V_{max} decrease should be different for the two reactions; in particular it should be greater for the hydration reaction where the substrate, namely fumarate, is aprotic and less polar than the product.

In the dehydration reaction, in contrast, the substrate, L-malate, is more polar than the product, fumarate, which contains a π-conjugated system and does not possess hydroxy groups. As a consequence both the access of the substrate to the enzyme and the release of the product into the reaction medium should be more favoured than in the hydration reaction. This is also suggested by the experimental fact that in 20% methanol the decrease of V_{max} with

respect to water is 79% for the hydration of fumarate and 67% for the dehydration of L-malate.

Fumarase stability in methanol was studied measuring its activity, in terms of initial rates, after different periods of incubation in the reaction medium with different percentages of methanol. Data are reported in Table 3 where the results show that a clear decrease of activity occurred as the percentage of methanol in the reaction medium was increased.

This inhibition by methanol was further investigated and (for the methanol solutions) a surprising reversibility of inactivation was observed after evaporating methanol from the reaction medium. From 20% and 30% methanol, after the evaporation of methanol, fumarase regained almost 100% of the activity in aqueous solution while from a 40% solution of methanol the final activity of fumarase after the evaporating process is 75% of the original activity in water. These results show that denaturation is always at least partially reversible. The activity restoration is completed only after a day from the regeneration of the pure aqueous medium, suggesting the occurrence of renaturation kinetics. The results of methanol evaporation are shown in Fig. 4. The loss of activity from fumarase solutions of 20% and 30% methanol does not seem to be due to irreversible denaturation, but was interpreted by us in terms of a partial subtraction of water, by the organic solvent, from the environment closest to the enzyme with consequent decrease of mobility of

Table 3
Fumarase stability in terms of V_0 as a function of methanol (v/v) percentage in the reaction medium

Days of incubation	V_0 (μmol l^{-1} s^{-1}) at different % of methanol		
	20	30	40
0	25.0	11.6	4.8
1	22.5	11.0	3.8
5	22.9	8.9	1.2
7	22.7	8.1	1.0

Conditions: 7.2 U/ml fumarase and fumarate 5 mM at 25°C.

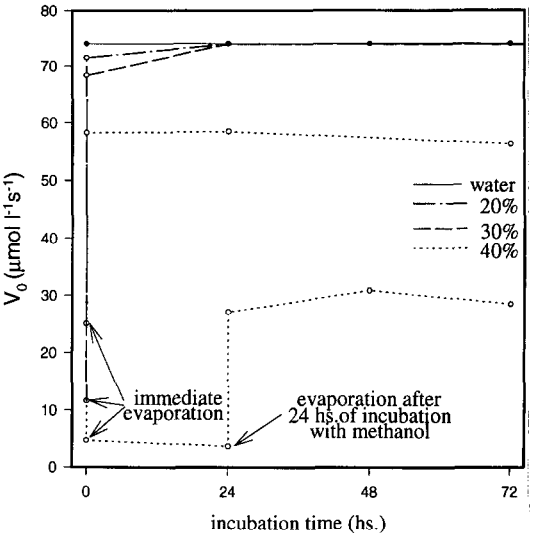

Fig. 4. Residual activity of fumarase solutions after the evaporation of methanol from the reaction medium.

the enzyme itself and consequent decreased efficiency.

The presence of phosphate buffer enhances the catalytic activity of fumarase, probably since it favours proton exchange between enzyme and solvent [7–9]. However since the water solution of the buffer was 50 mM, the phosphate concentration with respect to the reaction medium decreased as the percentage of organic solvent was raised. In order to better understand the effect of this decrease of phosphate concentration on the fumarase activity, the hydration reaction of fumarate was also carried out keeping phosphate concentration constant with respect to the overall reaction medium (by increasing its water concentration). The percentage of conversion vs. time with phosphate 50 mM with respect to the water/methanol systems is shown in Fig. 5 while the initial rates for different substrate concentrations are plotted in Fig. 6. The values of V_{max}, K_M and K_{eq} shown in Table 4 may be compared with those listed in Table 1. The action of phosphate anion seems to be activating at low concentrations and inhibiting at higher concentrations. This is also confirmed by the trend of the K_M: in 20% and 30% methanol the K_M is higher with 50 mM

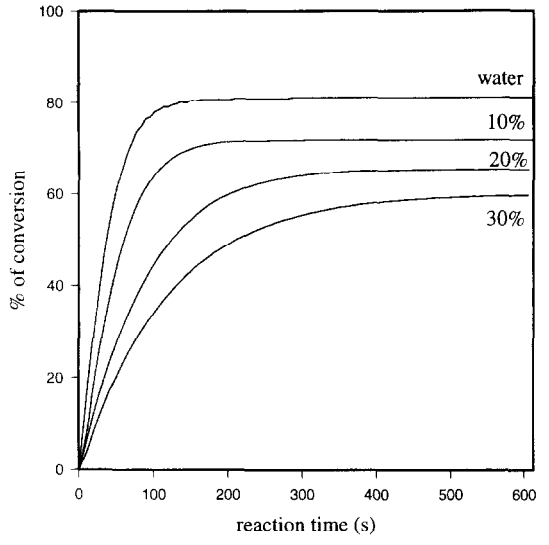

Fig. 5. Conversion vs. time of the hydration reaction of fumarate at different percentages of methanol in the reaction medium with phosphate at a constant concentration of 50 mM.

phosphate buffer (with respect to the reaction medium) than with 50 mM buffer with respect to the water content of the medium itself. This suggests that phosphate anions at higher concentrations may compete with the substrate anions for the active sites of fumarase, thus exerting an inhibiting action.

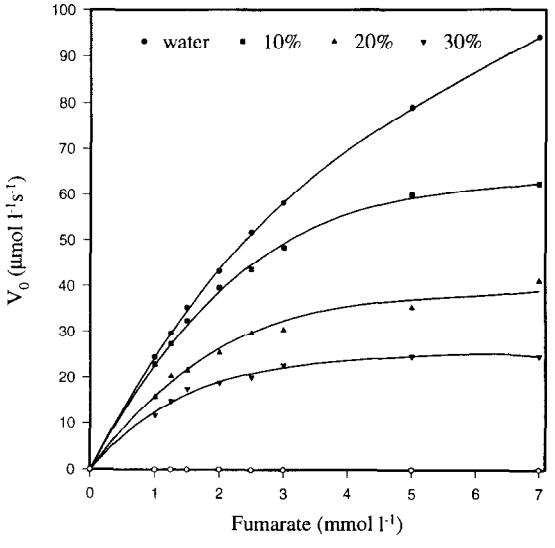

Fig. 6. Initial rate vs. fumarate concentration at different percentages of methanol in the reaction medium with phosphate at a constant concentration of 50 mM.

Table 4
V_{max}, K_M and K_{eq} values for hydration reaction of fumarate in water/methanol as a function of methanol (v/v) percentage in the reaction medium with phosphate buffer at constant 50 mM concentration

	water/methanol, as % methanol		
	10	20	30
V_{max} (μmol l^{-1} s^{-1})	99.9	54.8	37.1
K_M (mM)	3.28	2.34	1.98
K_{eq}	2.56	2.19	1.48

Conditions: fumarase = 7.2 U/ml, T = 25°C.

The fumarase activity for the hydration reaction of fumarate in water/formamide medium showed quite different characteristics with respect to water/methanol and other water/organic solvent mixtures [1]; formamide resulted much more denaturing than any of the other solvents studied so far. Even few minutes of incubation of the enzyme in a 15% solution of formamide were sufficient to cause a loss of activity while the enzyme was stable almost for a hour (the minimum time necessary to let the reaction reach equilibrium) only in formamide 10%.

The conversion vs. time in water and in formamide 10% is shown in Fig. 7 and the initial rates of the hydration of fumarate at different concentrations of substrate are shown in Fig. 8; Fig. 9 depicts the loss of activity of fumarase during the first minutes of incubation of the enzyme in the reaction medium with different amounts of formamide.

In 10% formamide V_{max} was 144.2 μmol l^{-1} s^{-1}, K_M to 4,71 mM and the equilibrium constant was 2.8. Out of the various mixtures with 10% of organic solvent formamide 10% resulted the least denaturing. A solution of fumarase prepared in 30% formamide was diluted, immediately after its preparation, up to an organic solvent content of 10%, taking care to get a final concentration of enzyme of 7.2 U/ml, and its activity was tested; however no recovery of the enzyme activity was observed.

It is evident that the nature of the reaction

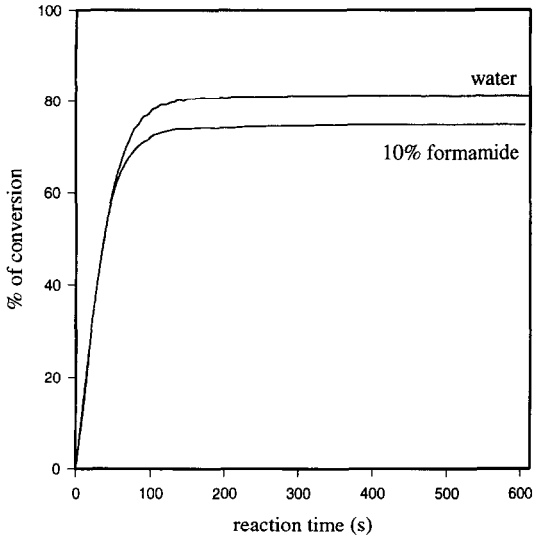

Fig. 7. Conversion vs. time of the hydration reaction of fumarate in water and in formamide 10%.

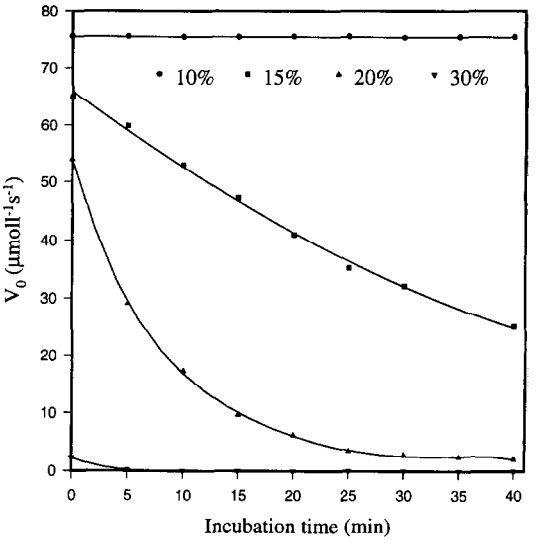

Fig. 9. Fumarase activity vs. time at different percentages of formamide in the reaction medium.

medium is able to alter both the kinetic and thermodynamic parameters of the biocatalysed reaction. The fact that the organic solvent decreases the enzyme activity may be explained on the basis of a denaturation of the protein with change in the protein conformation and consequent irreversible loss of activity. The data obtained with methanol however shows a reversible denaturation that points out the importance of the water molar fraction in the reaction media in order to guarantee a fully active conformation of fumarase.

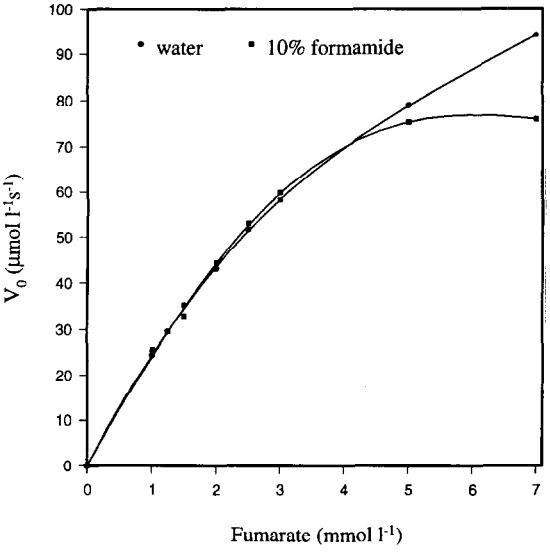

Fig. 8. Initial rate vs. fumarate concentration in water and in formamide 10%.

4. Conclusions

The further investigation of the catalytic behaviour of fumarase in non-conventional media has shown that, when methanol and formamide are used in mixture with water, a decrease of its activity is observed as the percentage of the organic solvent is increased.

At 10% of organic solvent the V_{max} is much higher with formamide than with methanol while this is not always true at higher percentages.

Fumarase stability is also influenced by the nature and percentage of organic solvent in the enzymic solution. A general decrease of activity is observed as the percentage of organic solvent increases. In 15% formamide the loss of activity is more than 60% after only 40 minutes of incubation in the reaction medium. With methanol solutions an unexpected reversibility of inactivation was observed, with a remarkable increase of activity after elimination of the organic solvent, while diluting solutions of formamide did not bring about any activity increase.

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