ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 1 (1996) 81–88

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

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

Enrico Cernia *, Riccardo Libori, Walter Marconi, Simonetta Soro

Department of Chemistry, University of Rome 'La Sapienza', Piazzale Aldo Moro 5, 00185 Rome, Italy

Received 29 June 1995; accepted 11 October 1995

Abstract

Fumarase catalysed hydration of fumarate was investigated in water/organic solvent one-phase systems. The organic solvents used were ethylene glycol, glycerol and dimethylformamide. 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 in all the reaction media. Together with a denaturing power of the solvent evidenced by a systematic decrease of V_{max} also a surprising decrease of the K_M was registered as the percentage of organic solvent in the reaction media was increased. While the equilibrium constant of the reaction (K_{eq} = [L-malate]/[fumarate]) decreased when the percentage of organic solvent was raised. An interpretation of these facts was given. Time-dependent denaturation was also investigated and glycerol resulted the less denaturing of the solvents used, while the aprotic DMF exhibited the highest deactivation.

Keywords: Fumarase; Organic solvents; Activity

1. Introduction

Fumarase, a homo-tetrameric protein, is a significant enzyme in the Krebs cycle of the tricarboxylic acids in mammalians [1–4]. It catalyses the reversible, stereospecific hydration of fumaric acid to L-malic acid, with an equilibrium constant of 4.2 at 25°C in a 50 mM phosphate buffer pH 7.3, which is the hydration of fumaric acid thermodynamically favoured in water. It is therefore present in every aerobic organism but it is also industrially utilised for the biotechnological production of L-malic acid [5–7]. It seems to be important to understand as fully as possible the reaction mechanism as well as the general catalytic properties of fumarase.

Recent applications of 'solvent engineering' to biocatalysed reactions offers a new tool in understanding enzyme mechanisms. Varying the composition of the reaction medium in enzymatic reactions also turned out to be a new way in elucidating the differences of affinity of the enzyme for its substrates and reaction products.

The organic solvents used for this study were chosen in a broad range of physical and chemical properties such as polarity, dielectric constant, density and so on, in order to permit, once mixed with water, the simultaneous solubilisation of enzyme, substrate and reaction products. Under these conditions the catalysis takes place in homogeneous phase. The organic solvents used in our study were: ethylene glycol, glycerol and dimethylformamide (DMF).

Kinetic and thermodynamic constants have been determined in every different reaction me-

* Corresponding author.

SSDI 1381-117(95)00276-6

dia in order to correlate their values with the variation in the composition of the reaction media and to interpret these data in terms of catalytic activity.

2. Materials and methods

Since fumarase is active only on the dianionic forms of its substrates [6], the salts of fumaric acid and L-malic acid were used for the experiments. Anhydrous fumaric acid disodium salt, 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 it was experimentally confirmed. All the organic solvents in the reaction media were from Farmitalia Carlo Erba (Milano, Italy).

2.1. Reaction kinetics

The hydration of fumaric acid is readily followed spectrophotometrically due to the absorption in the UV region of the double bond of fumaric acid, in the range 270–300 nm according to pH, solvent used and substrate concentration (Table 1 shows the extinction coefficients of fumarate).

Table 1
Fumarate extinction coefficients in phosphate buffer (50 mM, pH 7.3 at 25°C)

λ, nm	ϵ, l mol^{-1} cm^{-1}
230	4500
240	2230
250	1410
260	920
270	480
280	265
290	120

The measurements were made using a double beam spectrophotometer in which two 3 ml quartz cuvettes of 10 mm optical path can be placed. In one cuvette was placed 1 ml of fumarate solution at double the desired final concentration, to which was added 1 ml of fumarase solution in the same reaction medium, the other cuvette contained the blank of the reaction.

Absorbance values were recorded about 5 s after mixing substrate and enzyme at 25°C. The values of absorbance in the linear part of the kinetic curve are taken for initial rate for calculations. Eight different initial concentrations of fumarate were used to calculate V_{max} and K_M by the Michaelis–Menten method.

2.2. Reaction media

The aqueous reaction medium, used as a standard for comparing the fumarase activity was a 50 mM phosphate buffer solution, pH 7.3. The water/organic solvent mixtures were prepared by mixing the organic solvent under investigation at different percentages (v/v) with the 50 mM phosphate buffer solution, pH 7.3. These mixtures were then used to prepare both enzyme and substrate solutions.

In all the mixtures studied a simultaneous solubilisation of enzyme, substrate and reaction products was obtained. Fumarase solutions in the mixed solvents were prepared immediately before the measurements to avoid time denaturation.

3. Results and discussion

In order to compare the behaviour of our enzyme preparations in water/organic solvent and in pure water, the kinetic parameters in aqueous system were firstly investigated. The initial rate measurements of the dehydration reaction of L-malate, calculated for 8 substrate concentrations, were repeated at 3 different fumarase concentrations. From these experimental

Table 2
V_{max} and K_M values for dehydration of L-malic acid reaction as a function of enzyme concentration in phosphate buffer (50 mM, pH 7.3 at 25°C)

	[E] = 1.44 U/ml	[E] = 3.6 U/ml	[E] = 7.2 U/ml
V_{max} (μmol l^{-1} s^{-1})	19.7	59.2	116.2
K_M (mM)	14.94	15.70	15.32

data it was possible to calculate both V_{max} and K_M, as shown in Table 2, according to the Lineweaver–Burk interpolation; furthermore, from the relation $V_{max} = K_{cat}[E]$ the catalytic constant (K_{cat}) was calculated ([E] being the enzyme concentration) and its value was found to be equal to 0.0167 μmol s^{-1} U^{-1}. The equilibrium constant of the reaction, independent of enzyme concentration (K_{eq} = [L-malate]/[fumarate]) was 4.2 ± 0.1, corresponding to the range reported in the literature [8–11]. The hydration reaction of fumarate to L-malate was followed using 4 different initial concentrations of fumarase and the effect of enzyme concentration on both V_{max} and K_M was investigated. Table 3 reports the values of V_{max} and K_M. The trend of V_{max} was linear also for the hydration of fumarate and the value of K_{cat} of 0.0249 (definitely higher than for the dehydration of L-malate) was obtained to confirm a higher activity of fumarase for the direct hydration of fumarate. Moreover, the K_M for the dehydration of L-malate did not vary as the enzyme concentration increased, while the K_M for the hydration of fumarate increased with enzyme concentration showing a pattern of competitive inhibition. The values of the Michaelis–Menten constants however were generally lower for the hydration reaction, and this may be explained as a higher affinity of fumarase towards fumarate. As for the stability of the fumarase activity in water it was seen that the enzyme loses less than 4% of its original activity after 20 days of incubation in the aqueous solution. The disodium salts of fumaric and L-malic acids are insoluble in all of the 20 organic solvents tested (hexane, ethanol, tetrahydrofuran, acetone, diethyl ether, carbon tetrachloride, benzene, toluene, methylene chloride, dimethylsulfoxide, hexamethyl phosphoric triamide, acetonitrile, chloroform, dimethylformamide, ethylene glycol, glycerol, methanol, formamide, dioxane, trimethylamine) but they are soluble in water/organic solvent one phase systems at sufficiently high water contents. The hydration reaction of fumarate in water/ethylene glycol, studied using 8 different initial concentrations of fumarate, was carried out with a constant concentration of fumarase of 7.2 U/ml. The plots of initial rate vs. substrate concentration at each percentage of ethylene glycol are reported in Fig. 1 in comparison to the values obtained in pure water. The values of V_{max} and K_M obtained by the Lineweaver–Burk method

Table 3
V_{max} and K_M values for the hydration of L-malic acid reaction as a function of enzyme concentration in phosphate buffer (50 mM, pH 7.3 at 25°C)

	[E] = 1.98 U/ml	[E] = 3.96 U/ml	[E] = 5.94 U/ml	[E] = 7.92 U/ml
V_{max} (μmol l^{-1} s^{-1})	49.1	99.2	146.0	197.9
K_M (mM)	2.74	3.86	5.72	8.16

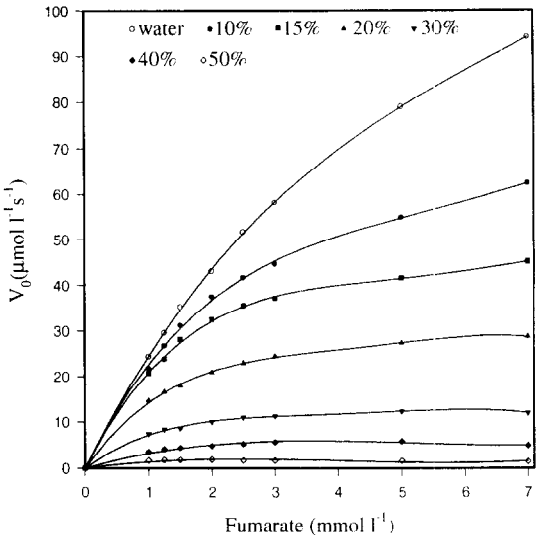

Fig. 1. Initial rate vs. fumarate concentration at different percentages of ethylene glycol in the reaction medium.

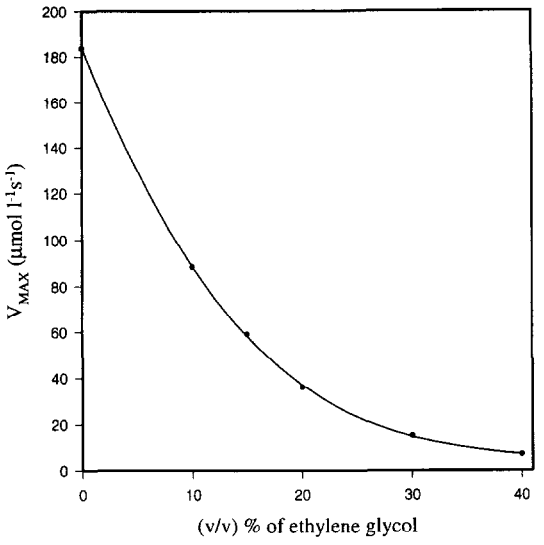

Fig. 2. Maximum velocity vs. percentage of ethylene glycol in the reaction medium.

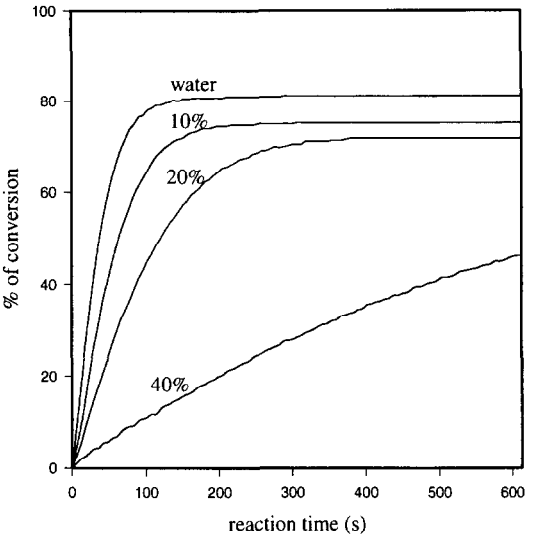

Fig. 4. Conversion vs. time of the hydration reaction of fumarate at different percentages of ethylene glycol in the reaction medium.

are plotted as functions of ethylene glycol percentage in the reaction media and are shown in Figs. 2 and 3, respectively, while the time/conversion plot is shown in Fig. 4.

Fumarase is not as stable in water/ethylene glycol as it is in water, indeed, in 40% ethylene glycol in a week it loses almost 40% of its activity. In Table 4 the activity of fumarase (in terms of initial rate) at different periods of incubation in water/ethylene glycol is shown.

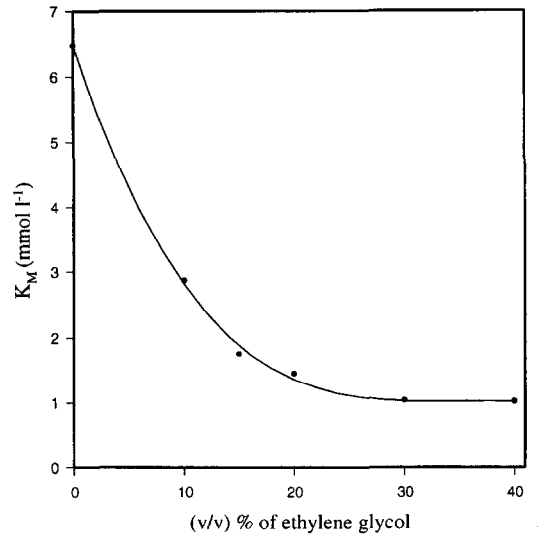

Fig. 3. Michaelis–Menten constant vs. percentage of ethylene glycol in the reaction medium.

The hydration reaction of fumarate to L-malate in water/glycerol mixtures at different percentages of glycerol in the reaction medium showed a similar trend for the parameters studied in water/ethylene glycol. Fumarase is generally less inhibited by glycerol than by ethylene glycol.

The initial rates vs. substrate concentration at different percentages of glycerol are plotted in Fig. 5. For each glycerol percentage both V_{max} and K_M were calculated and their trends as a function of glycerol content agree with the data obtained using ethylene glycol, although V_{max}

Table 4
Fumarase stability in terms of V_0 as a function of ethylene glycol (v/v) percentage in the reaction [a] medium

Days of incubation	V_0(μmol l^{-1} s^{-1}) at different % of ethylene glycol			
	10	20	30	40
0	52.6	27.4	12.3	5.8
3	51.4	23.4	7.9	4.9
7	50.2	17.6	5.2	3.6

[a] The hydration reaction was studied using 7.2 U/ml fumarase and 5 mM fumarate at 25°C.

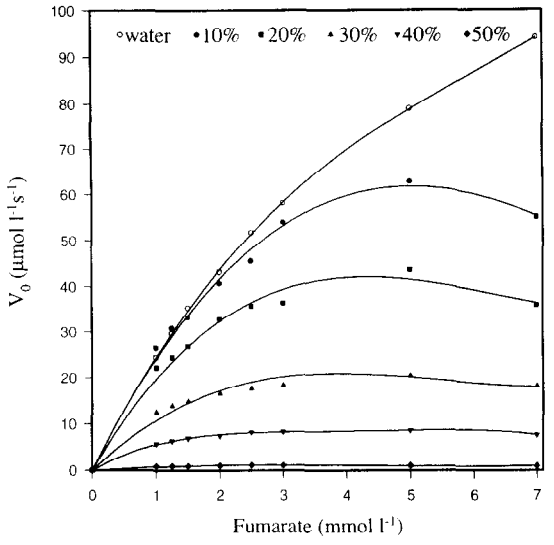

Fig. 5. Initial rate vs. fumarate concentration at different percentages of glycerol in the reaction medium.

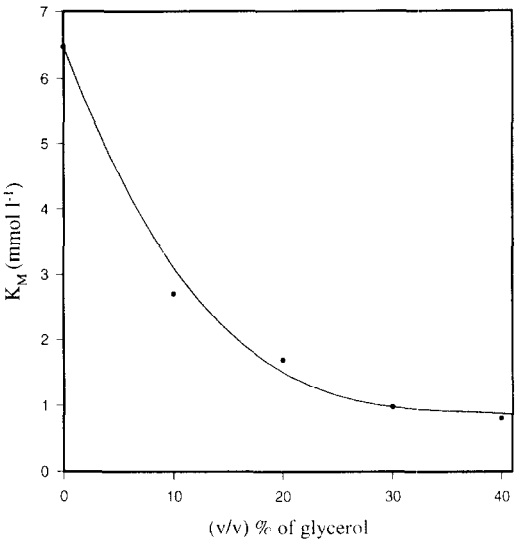

Fig. 7. Michaelis–Menten constant vs. percentage of glycerol in the reaction medium.

was always higher in glycerol than in ethylene glycol, showing a lower denaturing power of the former. Figs. 6 and 7 depict the values of both V_{max} and K_M in the glycerol solutions.

The equilibrium constant decreased as the percentage of glycerol in the reaction medium was increased showing a shift of the reaction towards the reagents.

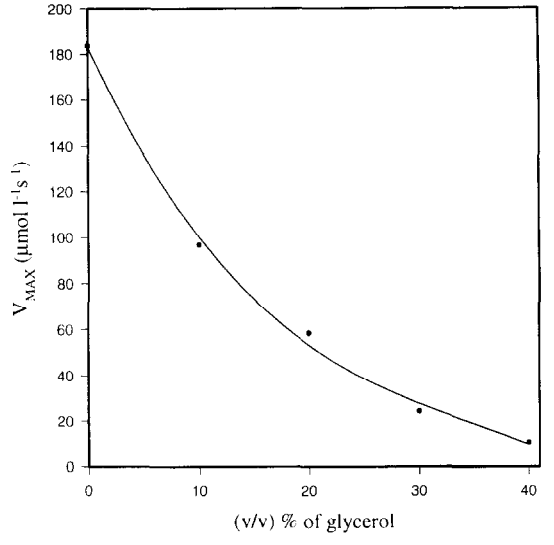

Fig. 6. Maximum velocity vs. percentage of glycerol in the reaction medium.

Fumarase activity in each reaction medium was tested at different incubation times of the enzyme in the solvent in order to detect a possible time-dependent denaturation. Table 5 shows the changes of V_0 after different incubation periods.

As can be seen from the table, glycerol denatures fumarase less than ethylene glycol, and in 10% glycerol this denaturation is almost absent, at least for the first 10 days. This lower denaturing power is also shown by the residual activity of fumarase in glycerol 50% with respect to ethylene glycol 50% in which the enzyme is fully inactivated.

The last organic solvent used for the preparation of a water/organic solvent mixture was

Table 5
Fumarase stability in terms of V_0 as a function of glycerol (v/v) percentage in the reaction [a] medium

Days of incubation	V_0 (μmol l^{-1} s^{-1}) at different % of glycerol				
	10	20	30	40	50
0	62.8	43.6	20.4	8.6	1.0
5	62.0	40.1	15.2	6.4	0.6
10	62.0	35.5	12.8	5.1	0.5

[a] The hydration reaction was studied using 7.2 U/ml fumarase and 5 mM fumarate at 25°C.

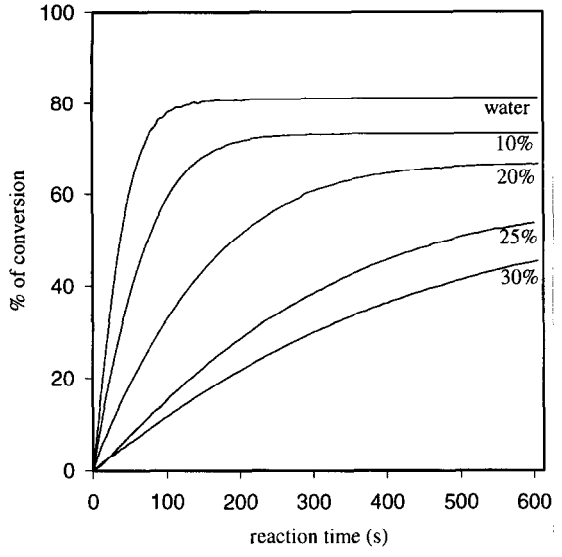

Fig. 8. Conversion vs. time of the hydration reaction of fumarate at different percentages of DMF in the reaction medium.

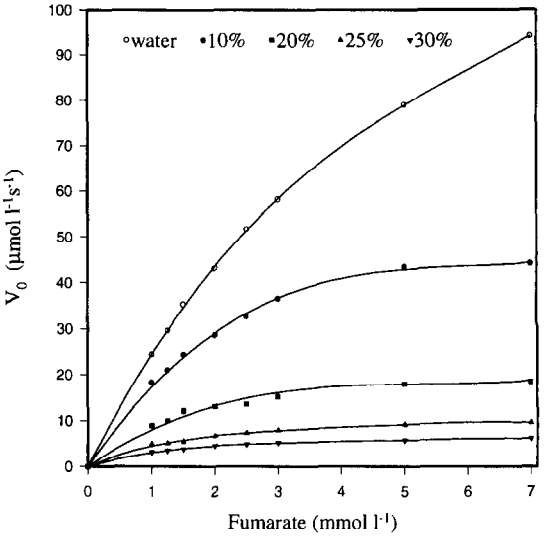

Fig. 9. Initial rate vs. fumarate concentration at different percentages of DMF in the reaction medium.

dimethylformamide which in contrast to the previous ones, is an aprotic solvent. Water/DMF mixtures were prepared with (v/v) percentages of DMF up to 30% and the biocatalysed hydration of fumarate was followed as described previously for ethylene glycol and glycerol. The conversion vs. time is shown in Fig. 8 where a sharp decrease of both initial rate and fumarate equilibrium concentration appears as the percentage of DMF in the reaction medium increases. The Michaelis–Menten plots of V_0 vs. [S] are shown in Fig. 9. With DMF the kinetic parameters V_{max} and K_M also decreased with percentage of organic solvent, though in this case the greatest denaturing effect was observed. The trends of V_{max} and K_M are depicted in Figs. 10 and 11. The equilibrium constant for the fumarate hydration reaction, Table 7, also reflects the general trend already observed with ethylene glycol and glycerol.

Dimethylformamide was the most strongly denaturing among the three solvents used, also in terms of time inactivation as determined (see Table 6) from the decrease of initial rate after some days of incubation at different percentages of DMF.

The interactions between enzyme, substrate and product are deeply affected by the composition of the reaction medium and by the chemical and physical properties of the solvent, affording affinity variations that can be inferred from the trend of the K_M. In all the reaction media a surprising decrease of the Michaelis–Menten constants is encountered when the percentage of the organic solvent in the reaction medium is

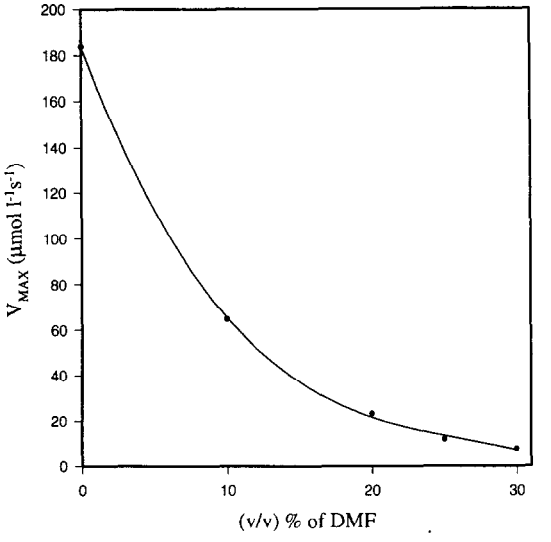

Fig. 10. Maximum velocity vs. percentage of DMF in the reaction medium.

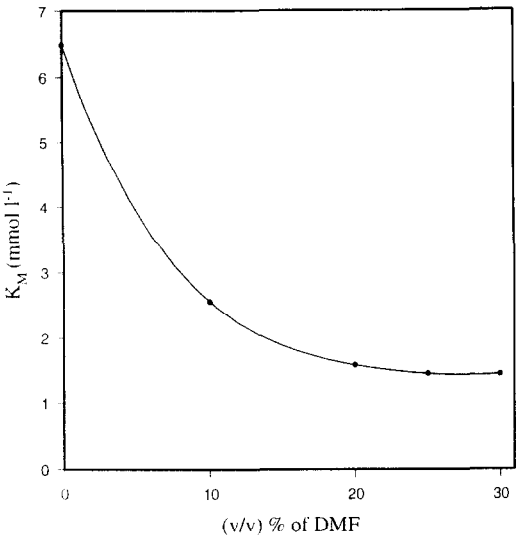

Fig. 11. Michaelis–Menten constant vs. percentage of DMF in the reaction medium.

increased, showing a higher affinity of fumarase for its natural substrates in non-conventional media than in water.

Indeed fumarase activity shows a strong dependence on the nature of the reaction medium. Even small percentages of organic solvent are sufficient to cause a dramatic fall of V_{max}, so in 10% ethylene glycol the V_{max} drops to half its value in water and falls to zero when the percentage of ethylene glycol reaches 50%. Glycerol is less denaturing than ethylene glycol which is less denaturing than DMF; since DMF is also the only aprotic solvent used, we think that this may be relevant in a reaction where proton exchange is involved in the rate-determining step, namely the regeneration of the enzyme in a free deprotonated form [12–14].

Table 6
Fumarase stability in terms of V_0 as function of DMF (v/v) percentage in the reaction [a] medium

Days of incubation	V_0(μmol l^{-1} s^{-1}) at different % of DMF		
	10	20	30
0	43.4	18.0	5.8
3	20.1	9.7	1.2
7	9.2	3.5	0.8

[a] The hydration reaction was studied using 7.2 U/ml fumarase and fumarate 5 mM at 25°C.

Table 7
Equilibrium constants (K = [L-malate]/[fumarate]) of the hydration of fumarate catalysed by fumarase at different (v/v) percentages of organic solvent in the reaction [a] medium

Ethylene glycol		DMF		Glycerol	
(v/v)	K	(v/v)	K	(v/v)	K
3	3.46	3	3.50	3	3.60
10	2.34	10	2.74	10	3.11
20	1.96	20	1.98	20	2.71
30	1.49	25	1.23	30	2.22
40	1.06	30	0.94	40	1.88

[a] The reactions were conducted with 7.2 U/ml fumarase and fumarate 5 mM at 25°C.

As for the equilibrium constant value in the different reaction media, we observed that it decreases with the increase of the amount of the organic solvent in the reaction medium (Table 7).

This reflects the different interaction energies of the products and reagents with mixtures containing different amounts of organic solvent. When the percentage of organic solvent is increased, the polarity of the medium varies, resulting more hydrophobic, and formation of fumarate, which is more hydrophobic than L-malate, is selectively favoured, as shown by the shift of the equilibrium constant. Moreover the nature of the reaction medium also influences the repartition of fumarate and L-malate between the available active sites and the reaction medium itself, and this plays an important role on the reaction kinetics, as shown by the decrease of K_M, as the result of an increase of affinity of the substrates (dianionic species) for the active sites of fumarase.

The generally observed diminution of V_{max} reflects a non-competitive inhibition by the organic solvent. This inhibition not only can be explained in terms of a conformational change due to interactions between the solvent and the hydrophobic/hydrophilic domains but also could originate by a loss of mobility of the enzyme when the amount of water decreases in

the region closest to the protein, affording a higher enzyme affinity towards the product.

4. Conclusions

In all the reaction media tested, a surprising decrease of the Michaelis–Menten constants was found when the percentage of the organic solvent in the reaction medium is increased, showing a higher affinity of fumarase for its natural substrates in the non-conventional medium than in water.

Fumarase activity shows a strong dependence on the nature of the reaction medium. Even small percentages of organic solvent are sufficient to cause a dramatic fall of V_{max}. This generally observed diminution of V_{max} reflects a non-competitive inhibition by the organic solvent. The equilibrium constant is also dependent on the amount of organic solvent in the reaction medium.

The possibility of varying the properties of the enzyme as well as the kinetic and thermodynamic parameters of biocatalysed reactions, by changing the nature of the reaction medium, appears to be a powerful tool in understanding reaction mechanisms and elucidating the affinities of an enzyme for its natural substrates, and we are carrying out further studies in order to obtain more detailed information about the molecular interactions between enzyme and reaction media of different nature.

References

[1] S. Yamato, Eur. J. Biochem., 93 (1979) 189–195.
[2] C.J. Sacchettini, J. Biol. Chem., 261, 32 (1986) 15183–15185.
[3] R.L. Hill, Brookhaven Symp. Biol., 17 (1964) 80–97.
[4] L. Kanarek J. Biol. Chem., 239 (12) (1964) 4207–4211.
[5] Kirk–Other, Encyclopedia of Chemical Technology, Wiley-Interscience, Chichester, 1993.
[6] Ullman's Encyclopedia of Industrial Chemistry, Vol. A13, Wolfgang Gerhartz, Weinheim, 1988, p. 515–517.
[7] I. Chibata, Methods Enzymol., 136 (1987) 455–463.
[8] R.L. Hill, Methods in Enzymol., 13 (1969) 91–99.
[9] D.A. Brant, J. Am. Chem. Soc., 85 (1963) 2204–2209.
[10] V. Massey, Methods in Enzymol., 1 (1969) 729–735.
[11] R.M. Bock, J. Am. Chem. Soc., 75 (1953) 1921–1925.
[12] R.A. Irwin. Biochemistry, 32 (1993) 8504–8511.
[13] R.A. Irwin. Biochemistry, 31 (1992) 9993–9999.
[14] J.S. Blanchard, Biochemistry, 19 (1980) 4506–4513.