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# Stability and activity of a thermostable malic enzyme in denaturants and water-miscible organic solvents

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A study was made of the effects of common protein denaturants and water-miscible organic solvents on both the stability and activity of the malic enzyme [(*S*)-malate:$NADP^+$ oxidoreductase (oxaloacetate-decarboxylating); EC 1.1.1.40] from the extreme thermoacidophilic archaebacterium *Sulfolobus solfataricus*. At 25°C, the enzyme was not inactivated in 4 M urea or 0.05% SDS over 24 h, while the half-life was 30 min in 6 M guanidine hydrochloride and 5 h in 0.075% SDS. The enzyme stability in water-miscible organic solvents at 25°C is somewhat surprising: after a 24-h incubation, the enzyme was completely active in 50% dimethylformamide; it lost 15% of its initial activity in 50% methanol or 15% ethanol. However, the resistance to organic solvents was greatly reduced at higher temperatures. The enzyme was able to catalyze the malate conversion even in the presence of 1.5% Triton X-100 or sodium deoxycholate. A number of solvents were found to stimulate the malic activity independent of time. Studies with 50% methanol revealed that the activation was reversible and inversely related to the temperature; moreover, the solvent was demonstrated to exclusively affect the maximal velocity of catalysis, the $K_m$ values for both substrates being unchanged. Investigation was made to find out whether there was a correlation between enzyme stability, as well as activation, and hydrophobicity of the organic medium. The residual malic activity after incubation in the water/organic medium correlated inversely with the logarithm of the partition coefficient in octanol/$H_2O$ of the mixture used as a hydrophobicity index. On the other hand, the extent of activation depended directly on the logarithm of the molar concentration of the organic solvent required for maximal enzymatic activation. Because of its remarkable resistance to organic solvents and protein denaturants in general, the malic enzyme from *Sulfolobus solfataricus* can be considered suitable for biotechnological applications.

Archaebacteria represent a third primary kingdom in addition to eukaryotes and eubacteria and are distinct from the latter by differences in their metabolic pathways and genetics [1]. In general, the enzymes isolated from the extreme thermophilic archaebacteria are thermostable and also more resistant to common protein denaturants and organic solvents than similar catalysts from mesophiles [2]. Investigation of how these unusual enzymes behave in nonaqueous solutions (i.e. the dependence of enzymatic properties on the nature of the solvent, kinetic parameters and conformational stability in organic solvents, etc.) can help to assess their structure/function stability relationship. On the other hand, the technological applications for enzymes in organic media are attracting more attention [3].

We have recently [4] purified to homogeneity and characterized a malic enzyme [(*S*)-malate:$NADP^+$ oxidoreductase (oxaloacetate-decarboxylating)] from the extreme thermoacidophilic archaebacterium *Sulfolobus solfataricus*, strain MT-4, grown aerobically at 87°C and pH 3.0, as previously described [5]. The malic enzyme had been discovered in pigeon liver by Ochoa et al. [6] and the same activity has been demonstrated to be widely distributed in eukaryotes and eubacteria. To date, the only other thermophilic malic enzyme isolated is from *Clostridium thermocellum*, by Lamed and Zeikus [7].

The malic enzyme from *S. solfataricus* catalyzes the oxidative decarboxylation of L-malate (a) and the decarboxylation of oxaloacetate (b), as follows:

$$\text{(a) L-malate} + \text{NAD(P)}^+ \xleftrightarrow{Mn^{2+}\text{ or }Mg^{2+}} \text{pyruvate} + \text{NAD(P)H} + CO_2$$

$$\text{(b) oxaloacetate} \xrightarrow{Mn^{2+}\text{ or }Mg^{2+}} \text{pyruvate} + CO_2 .$$

All the data reported in this paper are related to the reaction in (a) (malic activity). The enzyme, a dimer with a native molecular mass of 105 ± 2 kDa and apparently identical subunits, requires divalent metal cations for its action ($Mn^{2+} > Mg^{2+}$) and displays maximal activity at 85°C and pH 8.0. $NAD^+$ can replace $NADP^+$, although the latter is the preferred coenzyme. A remarkable property of this enzyme is its thermostability, the half-life for inactivation being 5 h at 85°C.

Here we report on the effects of some common protein denaturants (such as chaotropic agents and detergents) and water-miscible organic solvents on both the stability and ac-

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*Abbreviations.* log *P*, logarithm of partition coefficient in an octanol/water two-phase system; log $P_{solv}$, log *P* value for the organic solvent; log $P_{mix}$, log *P* value for the water/organic-solvent mixture; log *C*, logarithm of the molar concentration of the organic solvent required for maximal enzymatic activation; HLB, hydrophile-lipophile balance.

*Enzyme.* (*S*)-Malate:$NADP^+$ oxidoreductase (oxaloacetate-decarboxylating), malic enzyme (EC 1.1.1.40).

tivity of the *S. solfataricus* malic enzyme. In addition, we investigated the correlation between the hydrophobicity of the organic medium and the enzymatic behaviour: for this purpose, we used the logarithm of the partition coefficient for the water/organic-solvent mixture (log $P_{mix}$) as a quantitative measure of hydrophobicity.

## MATERIALS AND METHODS

### *Chemicals*

$NADP^+$, Tris (Trizma reagent), urea and guanidine hydrochloride were from Sigma; acetone, 2-propanol, methanol and tetrahydrofuran from Merck; ethanol, *n*-butanol and dimethylformamide from Baker; L-malic acid, sodium deoxycholate and Triton X-100 from BDH chemicals. The Bio-Rad protein reagent and SDS were from Bio-Rad laboratories. All other chemicals used were of the highest purity available.

Malic enzyme was purified to homogeneity from the extreme thermoacidophilic archaebacterium *Sulfolobus solfataricus*, strain MT-4, according to the published procedure [4].

### *Enzyme assay*

The oxidative decarboxylation of L-malate was followed with a Varian DMS-100 recording spectrophotometer by observing the appearance of NADPH at 340 nm. The standard reaction mixture (final volume, 1 ml) contained 20 mM Tris/HCl, pH 8.0, 1 mM L-malate (adjusted to pH 7.0 with KOH), 0.05 mM $NADP^+$, 0.1 mM $MnCl_2$, 50 mM ammonium sulfate and enzyme (5–10 μg, an amount that caused an increase in absorbance in the range 0.02–0.15 $min^{-1}$); the assay temperature was 60 °C except where otherwise indicated. The pH was always adjusted at the indicated temperature and measured experimentally using model mixtures having the same composition. All the initial velocity determinations were averages of measurements performed in duplicate or triplicate. One unit of enzyme activity was defined as the amount of enzyme reducing 1 μmol $NADP^+$/min under the assay conditions.

The protein concentration was determined by the Bio-Rad method [8] using bovine serum albumin as standard.

### *Enzyme stability*

The stability of the malic activity in the presence of a given water-miscible organic solvent was examined by incubating, in stoppered glass tubes at 25 °C and in some cases at 45 °C and 60 °C, a mixture containing the malic enzyme (about 150 μg) and the solvent to be tested in 20 mM Tris/HCl, pH 8.0, in a final volume of 0.5 ml. Immediately after the addition of the solvent, and at convenient time intervals, 30-μl aliquots were removed from each incubation mixture after mixing and these were assayed for enzyme activity as already described. The stability of the malic activity in urea, guanidine hydrochloride and SDS was carried out at 25 °C, essentially as described above for the stability in organic solvents. The reported results are expressed as percentages of residual activity as a function of the incubation time.

### *Enzyme activity in non-aqueous media*

The effects of increasing concentrations of organic solvents (5–70%) or detergents (0.01–1.5%) on malic activity were investigated at 25 °C and, in some cases, at 45 °C and 60 °C in standard assay conditions, using stoppered cuvets to avoid evaporation. The organic solvents were added in place of an equivalent volume of buffer (by vol.) and addition of the solvents was, assumed to result in strictly additive changes. The average error in the determination of the initial reaction rate was ±10% when the reaction mixture contained an organic solvent concentration in the range 60–70%. The slight changes in pH of the reaction mixture caused by the addition of the organic solvent [9] were ignored since the malic activity was found to be unchanged between pH 8.0 and 9.0 at any temperature [4]. The malic activity values are expressed as a percent of the control (100%) performed in the absence of solvent or detergent.

Log *P* was used as a parameter of hydrophobicity; *P* denotes the partition coefficient of a given solute in a standard water/octanol two-phase system. Log *P* values for organic solvents (log $P_{solv}$) and for the water (log $P_w$) were obtained from hydrophobic fragmental constants according to Rekker et al. [10] and Leo et al. [11]. Log *P* values for the water/organic-solvent mixtures (log $P_{mix}$) were calculated using the following semi-empirical formula [12]:

$$\log P_{mix} = (1\text{-}x) \log P_{solv} + x \log P_w,$$

where *x* is the water concentration (in mole fraction) [13] and log $P_w$ is the log *P* value for water, considered to be equal to −1.38.

### *Circular dichroism*

Far-ultraviolet circular dichroism spectra were measured by a Jasco J-500A automatic recording spectropolarimeter at 25 °C, with a temperature control system employing a Haake bath. Measurements were made with stoppered quartz cells of 1-mm path length. An enzyme concentration of 0.1 mg/ml was utilized.

## RESULTS

### *Stability of the malic activity in chaotropic agents and water-miscible organic solvents*

Fig. 1 shows the effects of urea, guanidine hydrochloride and SDS on the stability of the malic activity at 25 °C. The enzyme retained almost 90% of the initial activity after 24 h of incubation in 6 M urea and had a half-life of 10 h in 7.5 M urea; the enzyme was completely stable after 24 h in 4 M urea (not shown). Guanidine hydrochloride was a more potent inactivator than urea, the half-life being 30 min in 6 M. No loss of activity was detected after 24 h of incubation in the presence of 0.05% SDS, whereas the enzyme half-life was 5 h in 0.075% SDS; the enzyme lost all its activity within 2 h in 0.1% SDS (not shown).

The stability study in the presence of various water-miscible organic solvents was carried out at 25 °C (Fig. 2). When the enzyme was incubated in 10% (by vol.) 2-propanol, the time progress curve of the stability showed that the activity increased slightly reaching a maximum value after 6 h of incubation; when incubated in 30% 2-propanol, the enzyme appeared to inactivate with a half-life of 10 h. The enzyme had a half-life of 8 h in 50% ethanol, whereas it kept 85% of its initial activity after 24 h in 15% ethanol or 50% methanol. No loss of activity was detected after 24 h of incubation in the presence of a concentration as high as 50% dimethylformamide. The enzyme had a half-life of 30 min in 50%

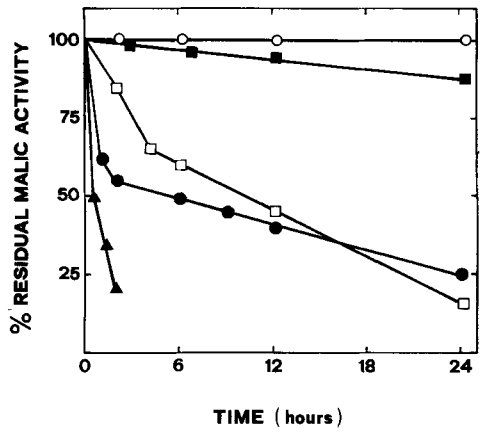


Fig. 1. *Effects of common protein denaturants on the stability of the* S. solfataricus *malic enzyme at 25°C.* The enzyme was incubated in stoppered glass tubes at 25°C in 20 mM Tris/HCl, pH 8.0, in the presence of the denaturing agent. At the times indicated, the residual malic activity was measured by the standard assay using 30-µl aliquots from each incubation mixture. (○) 0.05% SDS; (●) 0.075% SDS; (■) 6 M urea; (□) 7.5 M urea; (▲) 6 M guanidine hydrochloride

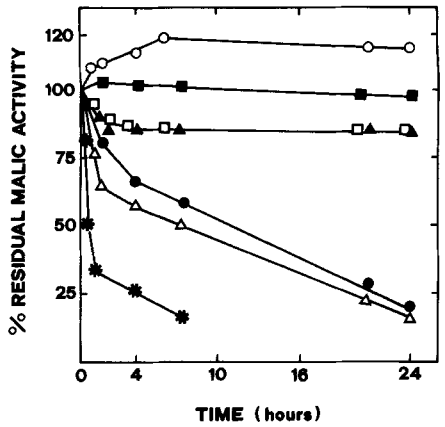


Fig. 2. *Effects of water-miscible organic solvents on the stability of the* S. solfataricus *malic enzyme at 25°C.* The conditions of incubation and assay of the residual activity were as in Fig. 1. All the solvent percentages were by vol.: (○) 10% 2-propanol; (■) 50% dimethylformamide; (▲) 50% methanol; (□) 15% ethanol; (●) 30% 2-propanol; (△) 50% ethanol; (★) 50% tetrahydrofuran

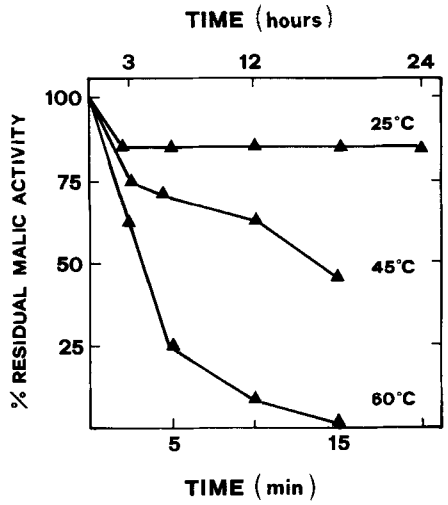


Fig. 3. *Effect of 50% methanol on the thermostability of the* S. solfataricus *malic enzyme.* The enzyme was incubated in stoppered glass tubes at 25°C, 45°C and 60°C in 20 mM Tris/HCl, pH 8.0, containing 50% methanol. At convenient time intervals, 30-µl aliquots were removed from each incubation mixture and assayed for the residual malic activity in standard conditions. In the time scale (hours) at the top of the figure, the inactivation was carried out at 25°C and 45°C; in the time scale (min) at the bottom the inactivation was carried out at 60°C

Table 1. *Effects of detergents on the activity of the* S. solfataricus *malic enzyme*
The malic activity was assayed at 25°C in 20 mM Tris/HCl, pH 8.0, containing 1 mM L-malate, 0.05 mM $NADP^+$, 0.1 mM $MnCl_2$, 50 mM ammonium sulfate and the detergent to be tested at the indicated concentration. Each point represents the mean of multiple measurements

| Addition | Concentration | Relative activity |
|---|---|---|
| | % | |
| None | – | 100 |
| Triton X-100 | 0.02 | 100 |
| | 0.5 | 90 |
| | 1.5 | 75 |
| Sodium deoxycholate | 0.2 | 100 |
| | 0.5 | 100 |
| | 1.5 | 95 |
| SDS | 0.05 | 85 |
| | 0.1 | 60 |
| | 0.3 | 0 |

tetrahydrofuran. The effect of 50% methanol on the thermostability of the malic activity was also investigated (Fig. 3); the enzyme half-life was 16 h and 3 min at 45°C and 60°C, respectively. When incubated at the same temperatures in the absence of the solvent, the enzyme retained all the activity over 12 h.

### *Effect of detergents and water-miscible organic solvents on the malic activity*

Since the activity of some thermophilic enzymes has been reported to be stimulated by detergents [14], we tested the effect of such compounds on the malic activity. Table 1 summarizes the results obtained at 25°C with increasing concentrations of neutral (Triton X-100) and ionic (sodium deoxycholate and SDS) detergents in the assay medium. There was no activation over the whole range of concentration (0.01 – 1.5%) for each detergent. Nevertheless, a correlation is likely between the hydrophilic/lipophilic balance (HLB) of the detergent, which is an inverse measure of hydrophobicity [15], and the effect exerted on the enzyme activity. In fact, the extremely hydrophobic detergents Triton X-100 (HLB = 13.5) and sodium deoxycholate (HLB = 16) appeared to affect in minimum extent the activity of the enzyme as compared to SDS (HLB = 40).

Fig. 4A depicts the effects of increasing concentrations of several water-miscible organic solvents on the malic activity at 25°C. All the solvents stimulated the initial rate of the malate conversion; however, they differed in the concentration required for maximal stimulation and the extent of

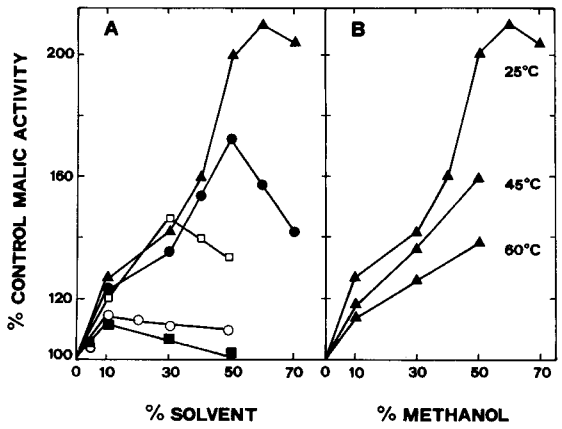


Fig. 4. *Effects of increasing concentrations of water-miscible organic solvents at 25°C (A) and methanol at 25°C, 45°C and 60°C (B) on the activity of the* S. solfataricus *malic enzyme.* The malic activity was assayed at the designed temperature in 20 mM Tris/HCl, pH 8.0, containing 1 mM L-malate, 0.05 mM NADP, 0.1 mM $MnCl_2$, 50 mM ammonium sulfate and the solvent to be tested, using stoppered cuvets. All the solvent percentages were by vol.; each point represents the mean of multiple measurements. (▲) Methanol; (●) ethanol; (□) acetone; (○) 2-propanol; (■) dimethylformamide

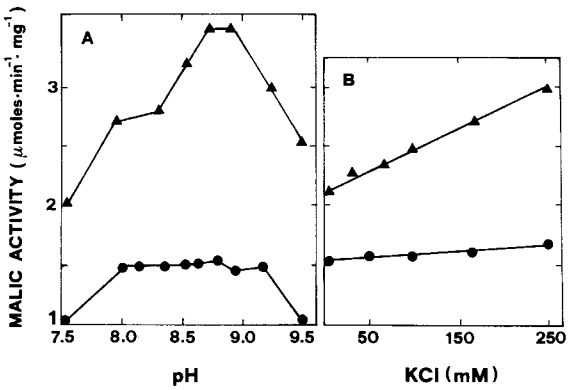


Fig. 5. *Dependence of the* S. solfataricus *malic activity on pH (A) and ionic strength (B) in the absence (●) and in the presence (▲) of 50% methanol in the reaction mixture at 25°C.* (A) The assay mixtures were standard except for the buffer employed: 20 mM Tris/HCl was used in the pH range 7.5–9.16, 20 mM glycine/NaOH was used for the point at pH 9.5. The pH values of the mixtures containing 50% methanol were corrected according to Bates [9]. (B) The standard mixtures contained concentrations of KCl in the range 0–0.25 M. The scale on the ordinate is valid for both A and B

Table 2. *Reversible enhancement of the activity of the* S. solfataricus *malic enzyme by methanol*
The enzyme was incubated at 25°C in 20 M Tris/HCl, pH 8.0, 50 mM ammonium sulfate, containing 50% ethanol for 10 min, then diluted in standard assay mixture at 25°C with or without 50% methanol

| Incubation conditions | Activity after dilution in | |
|---|---|---|
| | 0% methanol | 50% methanol |
| | $\mu mol \cdot min^{-1} \cdot mg^{-1}$ | |
| 0% methanol | 2.06 | 3.95 |
| 50% methanol | 2.04 | 4.02 |

activation. The most effective solvent for the rate enhancement was methanol. The rate of malate conversion increased almost linearly with methanol concentration, reaching a maximum at 60% (by vol.) methanol, which corresponds to a 1.1-fold rate enhancement. Stimulated levels of malic activity were still evident at a concentration as high as 70% methanol or ethanol. The other solvents showed a slighter activating effect at lower concentrations (acetone > 2-propanol ≈ dimethylformamide); however, they caused inhibition at relatively high concentrations (not reported in Fig. 4). Tetrahydrofuran and *n*-butanol did not display any activating effect (data not shown). No reduction of $NADP^+$ was observed in the presence of any solvent when the enzyme was excluded. The malate conversion proceeded linearly with respect to time, without an initial lag phase, in the standard aqueous medium as well as in the presence of an organic solvent.

Stimulation of the malic activity by methanol was completely reversible. The enzyme was incubated at 25°C in 20 mM Tris/HCl buffer, pH 8.0, 50 mM ammonium sulfate, containing 50% methanol, for 10 min, then diluted into the standard reaction medium at 25°C with or without 50% methanol; the enzyme activity returned to the control level when the solvent concentration was lowered to 0.3% (Table 2). Although not shown, no aggregation or disaggregation of the enzyme molecule was caused by 50% methanol, as determined by gel-filtration chromatography performed on a Superose 12 (Pharmacia) at 25°C, both in the absence and presence of the solvent.

### *Effect of temperature, pH and ionic strength; kinetic constants*

In order to shed some light on the role of organic solvents on the perturbation of the stucture/function relationship in the malic enzyme, we analyzed the dependence of the malic activity on temperature, pH and ionic strength in an organic medium. The solvent used was methanol since it had shown the highest activating effect. We tested the solvent effect on the catalysis at 45°C and 60°C, considering that the malic enzyme from *S. solfataricus* is a thermophilic enzyme. The data in Fig. 4B show the detection of activation by methanol at 25°C, 45°C and 60°C. It is noteworthy that the extent of enzyme activation is inversely related to temperature. The other solvents tested (ethanol and 2-propanol) behaved in a similar manner (not shown).

In agreement with previous findings [4], the malic activity in the standard aqueous medium did not vary with the pH ranging from 8.0–9.0; above pH 9.0 the activity rapidly decreased. When the initial reaction rate was investigated over the same pH range in non-aqueous mixtures containing 50% methanol, a progressive increase of the activity up to pH 8.9 was detected, although methanol stimulated the rate of malate conversion over the entire pH range tested (Fig. 5A). In the presence of 50% methanol, the malic activity displayed a slight change in the dependence on the ionic strength increase in the reaction mixture (Fig. 5B).

Linear Lineweaver-Burk plots were obtained both in the absence and presence of 50% methanol. The Michaelis constants for malate (71 μM) and $NADP^+$ (12.5 μM) did not change significantly in methanol, while the $V_{max}$ for both substrates doubled (data not shown).

## DISCUSSION

The resistance of a number of thermophilic enzymes to chaotropic agents and detergents is well documented and is interesting both for structural implications and biotechnological potentials [16]. The reported stability, of the malic enzyme from the extreme thermoacidophilic archaebacterium *S. solfataricus*, to inactivation by urea, guanidine hydrochloride and detergents is unusual even when compared to that of other dehydrogenases from thermophilic sources [17, 18].

In order to investigate correctly the effects of the water-miscible organic solvents on the behaviour of the malic enzyme from *S. solfataricus*, two distinct phenomena were considered: (a) the residual enzymatic activity in standard assay conditions after incubation for different lengths of time in a water/solvent mixture, termed stability; (b) the enzyme catalytic power when the solvent is incorporated into the reaction medium, termed activity.

The malic enzyme from *S. solfataricus* was found to possess a remarkable stability in certain water-miscible solvents: after 24 h of incubation at 25 °C, the enzyme did not lose its activity in 50% dimethylformamide and retained 85% of its initial activity in 50% methanol or 15% ethanol. Generally, for several mesophilic enzymes, inactivation is associated with denaturation [19], aggregation [20] or dissociation [21]. In the case of the *S. solfataricus* malic enzyme, 50% methanol did not cause aggregation or dissociation of the enzyme molecule. However, tolerance towards the organic solvent was reduced at temperatures above 45 °C. According to reports on the enzymes from thermophilic sources, the *S. solfataricus* malic enzyme can function in the presence of a notable amount of water-miscible organic solvents and a number of solvents have even been found to enhance the activity of this enzyme (see Fig. 4A). In any case, a bell-shaped curve was obtained, with the reaction rate at first stimulated and then decreased. In the case of alcohols, the activating effect followed the series methanol > ethanol > 2-propanol at the same concentration (by vol.). Since it is known that the denaturing power of alcohols on proteins is inversely related to the same series [22], we can hypothesize that at each solvent concentration we are measuring a compromise between the activation, the inhibition (due to the lateral alkyl chain of the alcohol) and, probably, other effects. Activation by methanol was immediate and reversible, involving a slight change in the pH optimum of the malic activity and in the dependence of activity on ionic strength. An investigation of the kinetic parameters in methanol was made. Lineweaver-Burk analysis revealed that 50% methanol doubled the $V_{max}$, whereas the $K_m$ for both $NADP^+$ and L-malate was unmodified. The absence of a change in the Michaelis constants demonstrates that there are no inhibition effects and the enzyme retains its substrate specificity. Also, the efficiency of the reaction (expressed by $k_{cat}/K_m$ ratio) was higher in methanol, with respect to the aqueous buffer, indicating that the stimulation is due to an increase of the turnover number of the enzyme.

The conformational status of the enzyme in 50% methanol was investigated by far-ultraviolet circular dichroism. The CD spectrum of the enzyme in organic solvent did not change significantly with respect to that in water. By inference, the secondary structure of the malic enzyme in 50% methanol remains the same (or nearly the same) as in aqueous solutions.

Since organic solvents change the solution polarity, we attempted to search for a correlation between the hydrophobicity of the medium and the reported data of stability and activity of the malic enzyme in nonaqueous environment.

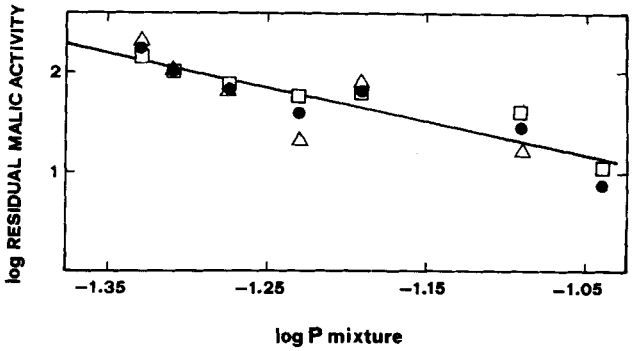


Fig. 6. *Influence of log* $P_{mix}$ *on the stability of the* S. solfataricus *malic enzyme in organic solvents.* The values of the residual malic activity at 1.5 h (□), 4 h (●) and 12 h (△) of incubation in the water-miscible organic solvent were taken from Fig. 2 and plotted versus the correspondent values of log $P_{mix}$ calculated as described in Materials and Methods. The log $P_{mix}$ values for 10% 2-propanol, 50% dimethylformamide, 15% ethanol, 30% 2-propanol, 50% methanol, 50% ethanol and 50% tetrahydrofuran were −1.337, −1.308, −1.275, −1.227, −1.188, −1.099, −1.037, respectively

We chose log *P*, the logarithm of the partition coefficient of a compound in a water/octanol two-phase system, as hydrophobicity parameter because it not only senses the differences in hydrophobicity between all common organic solvents, but well quantifies the hydrophobicity of mixtures differing in the water/organic-solvent ratio [23]. In this work, the log *P* values for organic solvents were obtained from hydrophobic fragmental constants [10, 11] and the log *P* value for each water/solvent mixture (log $P_{mix}$) was calculated taking into account the water concentration [12]. When the logarithms of the residual activity at 1.5 h, 4 h and 12 h of incubation in the presence of each solvent were plotted versus the corresponding log $P_{mix}$ values, an inverse correlation was found (Fig. 6). The higher its log *P* value, the more hydrophobic a mixture was: so, the enzyme stability decreased with the increase of hydrophobicity of the incubation mixture. An opposite enzyme stability/hydrophobicity relationship has been reported in certain two-phase systems [24]. A rationale is that miscible solvents strip or distort the water molecules at the protein surface which are required for stability and, when in contact with the protein, they exert a denaturing effect depending directly on their hydrophobicity; on the contrary, stabilization of the water layer by the immiscible solvents, (the more hydrophobic they are) can result in an increased thermal and storage stability of the protein [25]. Fig. 7 shows the logarithm of the solvent molar concentration which gave maximal stimulation of the enzyme activity (log *C*), versus the corresponding log $P_{mix}$ value; the relative activity value for each assay mixture is also reported. Two curves were thus obtained showing a similar behaviour; in fact, as outlined in the insert, a plot of the log *C* values versus the relative activity values was almost linear, thus suggesting the existence of a direct correlation between medium hydrophobicity and enzyme activation.

Activation by water-miscible organic solvents is not unknown in thermophilic enzymes [18, 26, 27], but very little information is available about the effects of such solvents on the catalytic and structural properties of these enzymes. The organic solvents may affect different protein molecules in a variety of different ways; so, it seems unlikely that a general explanation of the activation phenomenon can be found and

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