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Activity and stability of native and modified alanine aminotransferase in cosolvent systems and denaturants

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Abstract

Alanine aminotransferase (ALT) is used in clinical diagnostics, amino acid synthesis and in biosensors. Here we describe the stabilization of soluble porcine ALT by chemical modification with mono- and bis-imidates. The apparent transition temperatures ('T_m', the temperature where 50% of initial activity was lost in 10 min) for native and DMS-modified ALT were 46 and 56°C respectively. The effects of water-miscible organic solvents (methanol, dimethylformamide, dimethylsulphoxide and 1,4-dioxane) on the activity/stability of native and modified forms were determined. In all systems studied, an abrupt decrease in ALT catalytic activity was observed on reaching a certain threshold concentration of the organic solvent. The modified derivatives were more organotolerant than native enzyme. Comparison of the apparent V_{max} and K_m for 2-oxoglutarate as substrate, determined in 10% (v/v) organic solvent, with the results of thermal inactivation studies showed that the solvents have different effects on ALT's catalytic parameters and on its conformational stability. At 35°C with no organic solvent the dimethylsuberimidate (DMS)-modified derivative's half-life was 16 times greater than that for native enzyme; in 30% (v/v) solvent at 35°C, the DMS-modified ALT's half-life was up to 4.6 times greater than native enzyme's. DMS-modified ALT was also more stable in urea and guanidine HCl, and its refolding was more noticeable, than that of native enzyme.

Keywords: Alanine aminotransferase; Modification; Imidates; Organic solvents; Denaturants

1. Introduction

Alanine aminotransferase (ALT, EC 2.6.1.2) catalyzes the synthesis and breakdown of L-alanine by transfer of an amino group from the α-amino acid to 2-oxoglutarate. ALT has been used in biosensors together with lactate dehydrogenase to analyze lactic acid in several biological samples, such as pork meat [1], milk [2], or blood [3]; and in the production of L-alanine in native [4] and immobilized [5] form. It is also widely used as a clinical diagnostic marker of liver function [6] and is included in commercial control sera for clinical chemistry [7].

In these situations, ALT's long-term stability becomes an important characteristic. An enzyme loses activity due to unfolding of its polypeptide backbone (denaturation), which is reversible in many cases. Often, however, some other molecular process follows denaturation, leading to an irreversible loss of catalytic activity (inactivation) [8,9]. Enzyme stability is most

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often assessed by measurement of thermostability, since biocatalysts with high thermostability have a prolonged operational viability [10]. However, tolerance of organic solvents and of denaturants (such as urea and guanidine HCl) is also desirable. One can increase enzyme stability by methods as varied as protein engineering, immobilization, use of additives and chemical modification [9].

Here, we report the stabilization of ALT by chemical modification with the bifunctional compound dimethylsuberimidate (DMS), extending earlier work on beef ALT [11]. We focus in particular on ALT's resistance to the water-miscible organic solvents methanol, dimethylsulphoxide, dimethylformamide and 1,4-dioxane and to the denaturants urea and guanidine HCl. We also compare the effects of DMS with those of methyl acetimidate, a monofunctional analogue. Treatment with dimethylsuberimidate improves ALT's resistance to a variety of adverse influences.

2. Methods

Porcine heart alanine aminotransferase (ALT, lyophilized powder, 60 U mg^{-1} of solid) was obtained from Sigma, Poole, Dorset (UK) and was used without further purification. All chemicals for modification, urea and guanidine HCl were also from Sigma. Analytical grade organic solvents were supplied by Lab-Scan, Dublin (Ireland). All experiments were carried out in triplicate.

2.1. Enzymatic activity

ALT activity was measured by the continuous spectrophotometric IFCC (International Federation of Clinical Chemistry) method [12], where pyruvate produced in the ALT-catalyzed reaction is reduced by lactate dehydrogenase with oxidation of NADH to NAD^+. Enzymatic activity was monitored by following the decrease in absorbance at 340 nm in an ATI-Unicam UV–Vis spectrophotometer with thermostatted cuvettes (1 cm pathlength). Pyridoxal phosphate was included at a final concentration of 0.1 mM in the Tris/Alanine buffer, pH 7.5, as an activator. All reagents for this method were obtained from Sigma.

2.2. Imidates modification

The modification protocol was based on those of de Renobales and Welch [13] and of Minotani and colleagues [14]. Phosphate buffer 0.1 M and pH 8.0 was used to dissolve commercial ALT to a final concentration of 3 mg ml^{-1}. The enzyme solution was treated with either the monofunctional methylacetimidate (MAH) or with the bifunctional dimethylsuberimidate (DMS). These were added as dry powders, due to their instability in aqueous solution [15], at final concentrations of not less than 10 mM. The reaction took place at 25°C and, after 1 h, the reaction was terminated by the addition of an equal volume of cold 0.2 M Tris/HCl buffer, pH 7.0. Recovery of initial catalytic activity was estimated at the end of each experiment where unmodified ALT = 100%.

The degree of substitution was estimated from the spectroscopic measurement of remaining free NH_2 groups using trinitrobenzenesulphonic acid (TNBS) [16]. The degree of modification of ALT NH_2 groups was determined in relative units: absorbance of unmodified ALT corresponds to 100% of amino groups reacting with TNBS.

2.3. Temperature effect

Solutions (0.4 mg ml^{-1}) of native and modified enzyme were incubated in aqueous buffer for 10 min at temperatures between 4 and 70°C; the residual activity was then measured. The apparent transition temperatures, 'T_m', defined as the temperature where ALT activity declined over 10 min to 50% of its initial value, were estimated by inspection of plots of activity percentage versus temperature.

2.4. Effect of solvents

The residual activity of native and modified ALT was determined at 25°C in the presence of the organic solvents methanol (MeOH), dimethylformamide (DMF), dimethylsulphoxide (DMSO) and 1,4-dioxane (DXN). To 0.8 mg ml^{-1} solutions of native enzyme and of each modified form were added volumes of solvent to give overall solvent compositions between 0 and 90%. The mixtures were then placed at 25°C for 1 h after which time the residual ALT activity was measured as described above. Solvent remaining in the assay was $< 7.5\%$.

Solutions (0.4 mg ml^{-1}) of native and modified enzyme were also incubated at 35°C in the presence of a single concentration (30%) of organic solvent. Aliquots of each sample were withdrawn at various intervals onto ice until assay under the standard conditions above. Percent catalytic activity was determined for each time point relative to that of the appropriate sample at time zero. First-order rate constants (k) were calculated from fits of data points to a single exponential decay and the half-lives ($t_{1/2}$, the time required for activity to decrease to 50% of initial value) were estimated. The remaining solvent concentration in the assay was 2.5%.

2.5. Kinetic determinations

Kinetic determinations were carried out for the native and modified ALT in absence and presence of 10% of organic solvents over 50-fold range of 2-oxoglutarate concentrations according to the literature [12] at a constant concentration of 500 mM L-alanine. The V_{max} and K_m values were determined by fitting results to the Michaelis–Menten equation.

2.6. Unfolding / refolding test

The native and modified proteins (0.4 mg ml^{-1}) were incubated with different concentrations (0–5 M) of guanidine HCl (Gdn HCl) and urea in 0.1 M Tris/HCl buffer pH 7.5 for 1 h at 25°C. The residual activity was assayed at the end of each experiment. Activity in the absence of denaturant was considered as 100%.

Renaturation of the enzyme was studied after 60-fold dilution of the denaturant in the assay mixture. The native and modified enzymes were left in these conditions for 30 min at room temperature. After this time the activity was measured.

3. Results

3.1. Modification process and temperature effect

Table 1 shows the recoveries of catalytic activity following modification and the degree of modification of available lysines. The reactions resulted in little loss of enzyme activity and high degrees of modification. These results resemble those described earlier for beef ALT [11]. Values of the apparent 'T_m' are also shown in Table 1. These results indicate that the DMS-modified derivative is more heat-resistant than native enzyme. However, the MAH-modified derivative shows a 'T_m' lower than the native ALT's value. This decrease could arise from a deleterious effect of enzyme surface modification, as described in other cases [17,18].

3.2. Activity loss in organic solvents

For native and modified ALT, loss of activity in water-miscible organic solvents was investi-

Table 1
Results of the modification procedure and the effect of elevated temperature

Derivative	Residual activity (%)	Modification (%)	'T_m' (°C)
Native enzyme	100	0	46
DMS modified	92	77.5	56
MAH modified	97	82.5	42

'T_m' is defined here as the temperature where ALT catalytic activity decreases to 50% of its initial value in 10 min.

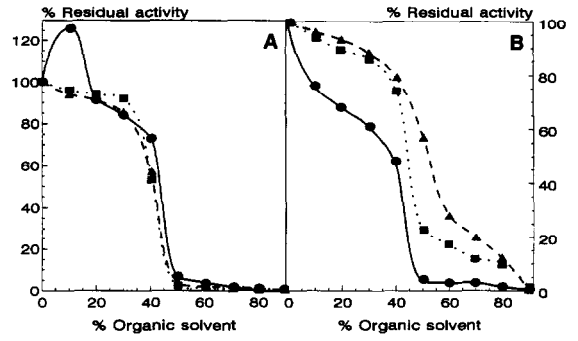

Fig. 1. Loss of ALT activity in: (A) methanol, (B) 1,4-dioxane. [Note that the left scale on the ordinate corresponds to graphic A and the right scale to graphic B.] Symbols: (—●—) native ALT; (· · ■ · ·) DMS-modified ALT; (– – ▲ – –) MAH-modified ALT.

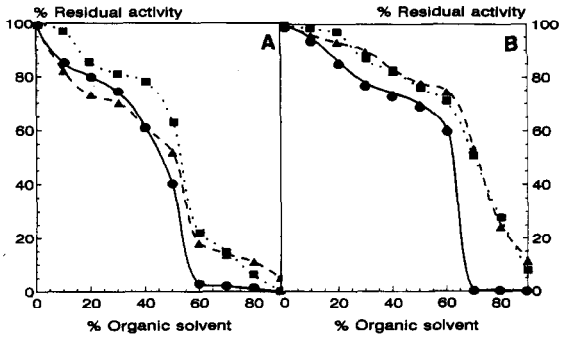

Fig. 2. Loss of ALT activity in: (A) dimethylformamide, (B) dimethylsulphoxide. Symbols: (—●—) native ALT; (· · ■ · ·) DMS-modified ALT; (– – ▲ – –) MAH-modified ALT.

gated: see Figs. 1 and 2. These indicate differences in ALT behaviour depending on the solvent type and concentration. Two ranges of concentration can be distinguished. In solvent concentrations up to 40%, enzyme activity decreases smoothly. This is followed by an abrupt drop in the activity of native and modified enzymes at higher concentrations (> 40%). This threshold loss of activity agrees with reports in the literature for other enzymes in presence of water-miscible organic solvents, i.e. in the irreversible inactivation of glucoamylase and invertase [19], or in studies of reversible denaturation of trypsin [20] and α-chymotrypsin [21].

The activity-loss profiles differ with solvent type. In MeOH–buffer mixtures (Fig. 1A) there are no notable differences in solvent resistance between the native and modified enzymes across the concentration range. However, native ALT appears to be activated at 10% MeOH. This was not observed for the modified derivatives. This may be due to MeOH-induced changes in the enzyme structure that do not occur in the modified derivatives.

In DXN (Fig. 1B) the native enzyme's activity decreases at low solvent concentrations. However, this diminution is much less in the case of either modified form. On the other hand, the MAH-modified enzyme is more stable at DXN concentrations > 50% than is DMS-modified ALT.

In DMF and DMSO (Fig. 2), loss of ALT activity also shows a threshold effect. Even at low solvent concentrations the enzyme activity decreases. Increasing solvent concentrations lead to complete inactivation. In DMF (Fig. 2A), the DMS-modified enzyme alone is slightly more tolerant than native ALT. In contrast, both modified forms are more tolerant of DMSO than native enzyme in the range 0–90% (Fig. 2B).

3.3. Inactivation curves

The time courses of the irreversible inactivation at 35°C in absence and presence of 30% (v/v) of each of the organic solvents were

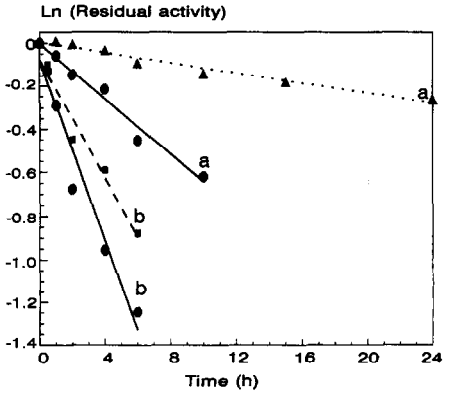

Fig. 3. Semilogarithmic decay plots for native and modified ALTs at 35°C, (a) in absence of solvent and (b) in 30% (v/v) DMF. Symbols: (—●—) native ALT; (· · ■ · ·) DMS-modified ALT; (– – ▲ – –) MAH-modified ALT.

Table 2
Deactivation constants of native and modified enzymes in presence of 30% organic solvent at 35°C

Derivative	Organic solvent	k [a] (h^{-1})	$t_{1/2}$ [b] (h)	Stabilisation [c] factor
Native	–	0.065±0.004	10.7	–
	MeOH	0.088±0.006	7.9	–
	DMF	0.243±0.030	2.9	–
	DMSO	0.054±0.004	12.8	–
	DXN	0.368±0.060	1.9	–
DMS modified	–	0.004±0.000 [d]	173.3	16.3
	MeOH	0.019±0.002	36.5	4.6
	DMF	0.148±0.021	4.7	1.6
	DMSO	0.017±0.001	40.8	3.2
	DXN	0.191±0.015	3.6	1.9
MAH modified	–	0.012±0.000 [d]	57.8	5.4
	MeOH	0.060±0.005	11.6	1.5
	DMF	0.225±0.007	3.1	1.1
	DMSO	0.023±0.001	30.1	2.3
	DXN	0.281±0.018	2.5	1.3

[a] Deactivation constants obtained by fitting the results to first order exponential decay equation.
[b] Half-lives (defined as $0.693/k$).
[c] Stabilisation factor, defined as the ratio between the k values of modified forms and of native enzyme.
[d] No error was detected in these values to three decimal places.

measured for the native and modified ALT. In all cases, the data fitted well to a single exponential decay equation (Fig. 3; for clarity, only some curves are depicted as examples) and the results are summarized in Table 2.

The DMS-modified enzyme in absence of organic solvent has a half-life 16 times greater than that of native ALT, while the MAH-modified derivative's half-life is only five-fold greater (Table 2). In presence of organic solvents, the stability differences due to the modifications are lower. In DXN, the DMS-modified derivative is almost twice as stable as native ALT. In DMF the DMS-modified ALT is more resistant than the native enzyme (Fig. 2A) but the stabilisation due to the MAH-modification is not significant. In MeOH, however, where the plot of residual activity against solvent concentration (Fig. 1A) showed parallel curves for the native and modified enzymes, the inactivation of DMS-modified ALT is slower ($k = 0.019$ h^{-1}) than those of the native enzyme and MAH-modified derivative (k values of 0.088 and 0.060 h^{-1} respectively). Inactivation of both modified forms in DMSO is also slower than that of native ALT (stabilization factors are 3.2 and 2.3 for DMS- and MAH-modified ALT respectively).

3.4. Kinetic constants

Table 3 shows the kinetic constants of native and modified enzymes in absence and presence of organic solvents at 10% (v/v). K_m of native enzyme for 2-oxoglutarate in buffer is similar to that described in the literature for pig heart ALT (0.4 mM) [22]. Variations due to the modification process are an insignificant increase in the K_m, and a diminution in the value of V_{max}, of modified derivatives with respect to the native enzyme. These differences can explain the diminished recovery of modified enzymes (Table 1), and are also shown in the diminished values of catalytic efficiency (Table 3).

In the presence of organic solvents, the V_{max}

Table 3
Apparent kinetic constants of native and modified enzymes

Derivative	Organic solvent	K_m (mM)	V_{max} (μkat/l)	Catalyic efficiency [a] (μkat/l mg mmol)
Native	–	0.411	3.11	1261
	MeOH	0.468	3.45	1228
	DMF	0.569	2.66	778
	DMSO	0.488	2.79	953
	DXN	0.796	2.19	458
DMS modified	–	0.443	2.72	1023
	MeOH	0.472	2.58	911
	DMF	0.476	2.63	921
	DMSO	0.447	2.70	1006
	DXN	0.481	2.54	880
MAH modified	–	0.462	2.83	1020
	MeOH	0.554	2.62	788
	DMF	0.486	2.53	868
	DMSO	0.483	2.73	942
	DXN	0.498	2.59	867

[a] Catalytic efficiency was defined as the relation between k_{cat} and K_m and was expressed as (μkat (l × mg of enzyme × mmol of substrate)$^{-1}$).

values of modified enzymes show a slight decrease while K_m increases. However, native ALT also shows a decreased V_{max} value and an increased K_m in the presence of solvents (especially in DXN) and, consequently, the catalytic efficiency is lower than in aqueous buffer. These variations in the kinetic parameters of native ALT correlate with the drastic reduction in the activity/stability of native enzyme observed in DXN (Table 2 and Fig. 1B).

3.5. Unfolding / refolding test

Both native and modified ALTs progressively lost activity at increasing concentrations of urea and guanidine HCl (Fig. 4). The activity loss was greater in guanidine HCl than in urea. Complete loss of activity occurred at 4 M guanidine HCl in all cases (Fig. 4A). In urea, native ALT lost most of its activity completely at 4 M denaturant but the modified derivatives retained 20% activity (Fig. 4B). Modified ALT is much more stable than native enzyme in 3 M urea. Less significant differences were found in the case of guanidine HCl. In both denaturants, ALT modified with DMS is slightly more stable than the MAH-modified derivative.

On dilution of the denaturant, varying percentages of the 'lost' activity could be recovered (Table 4). This refolding process is more noticeable in the DMS-modified derivative than in the native enzyme or MAH-modified derivative.

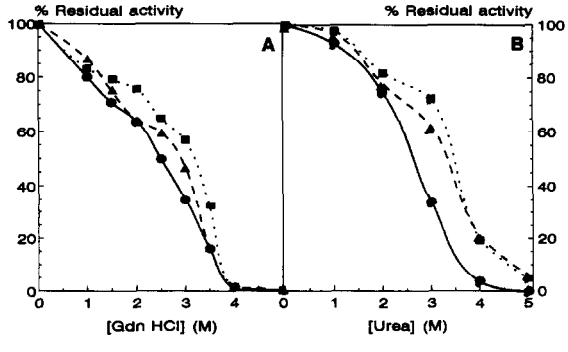

Fig. 4. Loss of ALT activity in: (A) guanidine HCl, (B) urea. Symbols: (—●—) native ALT; (· · ■ · ·) DMS-modified ALT; (– – ▲ – –) MAH-modified ALT.

Table 4
Recovery of activity of native and modified ALT after dilution of denaturant

Derivative	Agent	[Denaturant] [a] (M)	Activity recovered (%)
Native	Urea	1	98
		2	80
		3	42
Native	Gdn HCl	1	87
		2	75
		3	50
DMS Modified	Urea	1	100
		2	96
		3	90
DMS Modified	Gdn HCl	1	99
		2	94
		3	90
MAH Modified	Urea	1	100
		2	99
		3	93
MAH Modified	Gdn HCl	1	92
		2	80
		3	64

[a] Denaturant concentration before 60-fold dilution in assay mix (see Methods for further details).

4. Discussion

We have studied the catalytic properties of native ALT and of two imidate-modified forms in various water/organic solvent homogeneous mixtures. Treatment of native ALT with increasing concentrations of organic solvents eventually led in all cases to a profound loss of enzymatic activity, while the resulting modified derivatives, especially that obtained with DMS, are more organotolerant (Figs. 1 and 2). These losses may be caused by reduction of the active enzyme concentration (by a reversible or irreversible process) and/or by changes in the kinetic parameters. To differentiate between these two possibilities, the kinetic parameters K_m and V_{max} were determined for native and modified

Table 5
Physicochemical properties of organic solvent

Solvent	ε [a]	$E_T(30)$ [b] (kJ/mol)	log P [c]	DC [d]	α [e]	β [f]	π [g]
MeOH	33.3	232	−0.74	30.5	0.93	0.02	0.60
ACN	37.6	192	−0.34	64.3	0.19	0.31	0.76
DMSO	45.0	188	−1.35	60.3	0.00	0.076	1.00
DMF	37.7	183	−1.01	63.3	0.00	0.69	0.88
THF	7.9	156	0.46	100.0	0.00	0.55	0.58
DXN	2.9	151	−0.27	92.1	0.00	0.37	0.55

[a] Dielectric constant at 20°C [23].
[b] Empirical parameter for the solvent polarity [24].
[c] Logarithm of the partition coefficient of the solvent in a standard octanol–water two phase system [28].
[d] Denaturation capacity of the solvents [21].
[e] α scale of solvent (hydrogen donor) acidity describes the ability of the solvent to donate a proton to the solute [29].
[f] β scale of hydrogen acceptor basicity provides a measure of the solvent ability to accept a proton from the solute [29].
[g] π scale is an index of solvent dipolarity/polarizability which measures the ability of the solvent to stabilize charges of dipoles by its dielectric effect [29].

ALT under conditions where solvent-induced activity losses were negligible (Table 3).

The results for native ALT show that V_{max} increases in 10% MeOH and, since $V_{max} = k_{cat}[E]$, the active enzyme concentration is not reduced due to solvent. Therefore, the reduced activities observed in MeOH (except at 10%, Fig. 1) are likely due less to denaturation/inactivation but more to the observed increases in K_m. However, in 10% (v/v) DXN the K_m value increases (Table 3), reflecting a reduced affinity for the substrate due to the changed reaction medium. V_{max} also decreases in DXN. This reduction may arise from a decrease in k_{cat}, or from denaturation/inactivation.

In DMSO and DMF, K_m increases (but less than in DXN) and V_{max} is slightly reduced. This behaviour of the various solvents correlates with their denaturation capacity (DC), as described by Khmelnitsky et al. [21] (Table 5). In this scale, DXN has a 92.1% DC while MeOH has only 30.5% and DMSO and DMF are mid-scale [21]. On the other hand, solvent polarity plays an important role in substrate binding: the lower the $E_T(30)$ value [23,24] (Table 5), the lower the affinity (higher K_m). The $E_T(30)$ value is an empirical parameter for solvent polarity and is directly related to the free energy (ΔG) of the solvation process [23]. The solvatochromic $E_T(30)$ value describes the ability of different solvents to solvate polar fragments. The solvatochromic method measures changes in the molecular structure of a dye which represents changes in the three-dimensional protein structure due to solvent–protein interactions [24]. Other solvent polarity parameters (Table 5) consider only a part of the interactions, e.g., the dipole moment (which describes the polarization displacement and orientation) and the dielectric constant (the ability of a solvent to separate electrical charges).

In the modified ALT forms, regardless of the organic solvent, V_{max} decreases slightly while K_m increases relative to that in aqueous buffer. This indicates that, for modified ALT, changes in the catalytic parameters are more important than denaturation/inactivation due to organic solvent contact. The activity losses, as shown by the catalytic efficiency values (Table 3), are less than in native ALT.

On the other hand, the activity of native enzyme in 10% solvents correlated directly with the Dimroth–Reichardt parameter [23,24], $E_T(30)$ (Fig. 5). Experiments with two extra organic solvents (tetrahydrofuran (THF) and acetonitrile (ACN)) were carried out to analyze this correlation further (data not shown). When the maximum velocities of native enzyme (Table 3) were plotted against the $E_T(30)$ values of

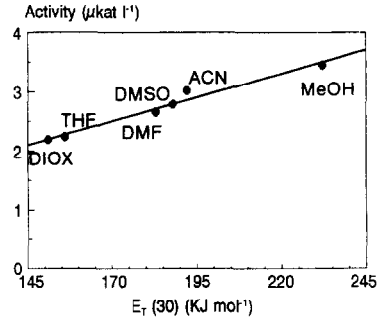

Fig. 5. Relationship between the activity of native enzyme in 10% of organic solvent and the Dimroth–Reichardt parameter [23,24].

each solvent (Table 5), the resulting line (Fig. 5) fit the equation:

$$A = 0.016E_T(30) - 0.24 \quad (r = 0.985)$$

where A is the activity of native ALT in 10% (v/v) water-miscible organic solvent in μkat l^{-1}, and $E_T(30)$ is the Dimroth–Reichardt parameter of solvent polarity in kJ mol^{-1}. Similar results have been reported for cytochrome c in presence of water-miscible organic solvents [18].

In addition to determination of activity and catalytic constants, ALT's thermostability in 30% (v/v) solvent was tested. Both native and modified enzymes showed a first-order exponential inactivation (Fig. 3). First-order exponential decay has also been found in other enzymes [18,19]. In monophasic water–organic solvent systems, enzymatic activity is normally lost at high solvent concentration by replacement of water in the protein's hydration surface layer by the organic solvent [25]. Displacement of bound water molecules by organic cosolvents results in a dramatic change of protein structure, leading to denaturation or inactivation [26].

From Table 5, the activity/stability of native and modified ALT in presence of organic solvents relates to the Dimroth–Reichardt parameter, $E_T(30)$ [23,24] (Fig. 5), and to the denaturation capacity of the solvents, DC [21]. (Note that $E_T(30)$ has been used for the determination of the denaturation capacity of solvents in the mathematical model of Khmelnitsky et al. [29].) No correlation could be found with any other parameter. Here, the stability differences between the native and modified enzyme increase when $E_T(30)$ decreases or DC increases.

Comparison of the results in Table 2 with those in Table 3 show that solvent effects on enzyme activity and enzyme stability are not equivalent. This means that the catalytic parameters and the active enzyme conformation differ in sensitivity to a solvent. Therefore, the choice of solvent as a medium for enzymatic reactions requires separate analysis of its denaturing effects and of its influence on enzyme kinetics.

Treatment of porcine heart ALT with imidates clearly results in increased tolerance of organic solvents. This modification-induced stabilization was also observed when native and modified ALTs were treated with denaturants (Fig. 4). Refolding following dilution of urea or guanidine HCl was more successful in the case of modified enzyme (Table 4).

Finally, if we compare both modified forms we observe that DMS-modified ALT is more stable in all cases than the MAH derivative. In both cases, the chemical reaction occurs through protein amino groups with retention of the positive charge [27]. However, DMS can form crosslinks of 11 Å length, while MAH is a functional group modifier and not a crosslinker. The differences are most noticeable in the apparent transition temperatures ('T_m' values, Table 1). DMS-modified ALT has a 'T_m' 10°C greater than native enzyme, while the MAH derivative's 'T_m' is 4°C below the native enzyme's. This suggests that modification with dimethylsuberimidate rigidifies the protein backbone by crosslinking, preventing unfolding (denaturation) and improving refolding of the enzyme molecule.

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