Further thermal characterization of an aspartate aminotransferase from a halophilic organism

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Aspartate aminotransferase (AspAT, EC 2.6.1.1) from the halophilic archaeobacterium *Haloferax mediterranei* was purified [Muriana, Alvarez-Ossorio and Relimpio (1991) *Biochem. J.* 278, 149–154] and further characterization of the effects of temperature on the activity and stability of the halophilic AspAT were carried out. The halophilic transaminase is most active at 65 °C and stable at high temperatures, under physiological or nearly physiological conditions (3.5 M KCl, pH 7.8). Thermal

inactivation (60–85 °C) of the halophilic AspAT followed first-order kinetics, 2-oxoglutarate causing a shift of the thermal inactivation curves to higher temperatures. The salt concentration affected the thermal stability of the halophilic transaminase at 60 °C, suggesting that disruption of hydrophobic interactions may play an important role in the decreased thermal stability of the enzyme.

INTRODUCTION

Enzymes from halophilic bacteria are characterized by the requirement for a high salt concentration to maintain their physicochemical properties. The stability and maximum activity of these enzymes are usually reached only with a high salt concentration, e.g. about 3 M KCl or NaCl [1,2]. In addition, some halophilic enzymes have a remarkable degree of thermophilicity, e.g. incubation of the halophilic dihydrolipoamide dehydrogenase at 95 °C for 15 min in the presence of 4 M NaCl resulted in no detectable loss of enzyme activity [3]. This unusual thermal stability of the halophilic enzymes has been studied mainly with dehydrogenases in pure or nearly pure states [4–8].

In an earlier report we described the purification of the halophilic aspartate aminotransferase (EC 2.6.1.1; AspAT) from *Haloferax mediterranei*, which not only tolerates salt for its stability, but also requires a high salt concentration for its activity [9–13]. This halophilic transaminase is a dimeric enzyme containing identical subunits; its coenzyme is weakly bound at each active site (K_d approx. 7 μ M) and in physiological conditions is found in equilibrium between the apo and the holo form inside halophilic cells [10,12]. This is not surprising as AspAT from higher organisms releases the coenzyme in a 1 M phosphate solution [14]. The aim of this study was to characterize further some thermal features of the halophilic AspAT. We report here a brief differential scanning calorimetry (DSC) study to detect and follow thermally induced order–disorder transitions, in the purified halophilic enzyme under different salt concentrations. Kinetic studies on the effects of substrates and the ionic strength of the medium at different temperatures were also carried out to determine whether or not ionic interactions influenced the thermal activity and stability of the halophilic AspAT.

MATERIALS AND METHODS

Bacterial strain and growth conditions

Haloferax mediterranei strain R-4 ATCC 33500 isolated from seawater-evaporation ponds near Alicante (Spain) was grown aerobically in liquid shake culture as described previously [15].

After 4 days of growth, the cells were collected by centrifugation and resuspended in a medium containing 20 mM sodium phosphate (pH 7.8) and 3.5 M KCl.

Enzyme purification

The cells were broken by pressure shearing, followed by ultrasonic disintegration as reported previously [16]. Cell debris was removed by centrifugation at 167 000 *g* for 30 min at 4 °C. The halophilic AspAT was then prepared from the supernatant according to our method [10]. The purified halophilic AspAT was judged to be homogeneous by the criteria of native and SDS/PAGE. The enzyme also sedimented as a single symmetrical peak during the sedimentation-velocity experiments [10].

Assay of enzyme activity and protein determination

The halophilic AspAT activity was measured at 25 °C by monitoring the oxidation of NADH at 340 nm in a reaction mixture (1 ml) which comprised 20 mM sodium phosphate buffer, pH 7.8, 3.1 M KCl, 10 mM 2-oxoglutarate, 0.3 mM NADH, 60 units of malate dehydrogenase from pig heart in 20% (v/v) glycerol, 0.05 mM pyridoxal 5'-phosphate, 100 mM L-aspartate, and an appropriate amount of enzyme. As a control, the stability of malate dehydrogenase from pig heart was tested under experimental conditions. This coupling enzyme was stable under the described conditions. Furthermore, the other components of the coupled assay for AspAT activity were also stable under the same conditions. Only at temperatures over 80 °C is it possible to detect a decrease in the oxaloacetate stability; however, its decomposition rate is lower than the rate for its conversion into L-malate. One unit of activity was defined as the amount of enzyme catalysing the conversion of 1 μ mol of substrate per min under the specified conditions. Protein was determined by the procedure of Lowry et al. [17], with crystalline BSA as standard.

Reconstitution of halophilic holoenzyme

As the apo form of the halophilic AspAT is obtained by our purification method [10], holoenzyme was prepared by reaching

Abbreviations used: AspAT, aspartate aminotransferase (EC 2.6.1.1); DSC, differential scanning calorimetry; ΔC_p , differential heat capacity; T_d , denaturation temperature.

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complete saturation of the native apoenzyme with 0.05 mM pyridoxal 5'-phosphate in darkness within 30 min.

DSC analysis

Calorimetric measurements were made on a Microcal model MC-2. Reference and sample volumes of 1.19 ml were used. All samples were dialysed against 50 mM sodium phosphate buffer, pH 7.8, at indicated salt concentrations; this solution was also used as the reference material. Both the sample and reference were de-aerated under reduced pressure for 15 min before delivery into the cells. After temperature equilibration of the assembly under vacuum, the heat capacity of the sample versus the appropriate reference solution was measured between 10 and 100 °C at a scanning rate of 1 °C/min. Protein concentrations ranging from 0.8 to 1.0 mg/ml were used. The calorimetric data were analysed as previously described [18–20]. The calorimeter directly monitors the differential heat capacity (ΔC_p), in calories per degree, of the sample cell with respect to the reference cell, as a function of temperature. Absolute values of the heat flow were obtained by using a calibration pulse which is a design feature of the instrument. The denaturation temperature (T_d) was obtained as the temperature of the maximum rate of heat flow in the thermogram. The calorimetric or real enthalpies for the denaturational transition ($\Delta_d H_{cal}$) were obtained by relating the area of the heat absorption peak to the area of a calibration mark. The values of $\Delta_d H_{cal}$ were then normalized per mol of protein.

Measurement of thermal activity and steady-state kinetics

In order to obtain the energies and entropies of thermal activation of the halophilic AspAT at 20–65 °C, we used the following Arrhenius and transition-state relationships [21,22]:

$$k_n = Ae^{-E_a/RT} \quad (1)$$

$$\Delta H^* = E_a - RT \quad (2)$$

$$k_t = \frac{k_b T}{h(e^{-\Delta G^*/RT})} \quad (3)$$

$$\Delta G^* = \Delta H^* - T\Delta S^* \quad (4)$$

The apparent energies of activation (E_a) for the thermal activation of the halophilic AspAT were calculated from Arrhenius plots of the variation of the Napierian logarithm of measured reaction velocities ($\ln k_n$) with the absolute temperature (T^{-1}) (eqn. 1), where R is the universal gas constant and A is the frequency factor. Activation enthalpy (ΔH^*) values at each temperature were calculated by using eqn. (2). Activation free energy (ΔG^*) values were calculated from eqn. (3), where h is Planck's constant, k_b is the Boltzmann constant, and k_t is the molecular activity for the halophilic enzyme. Activation entropy (ΔS^*) values were then calculated from eqn. (4).

Apparent standard enthalpies for substrate binding (ΔH_s) were estimated from plots of the variation in $\ln K_m$ versus T^{-1} , according to the van't Hoff relationship:

$$\partial \ln K_m / \partial T = -(\Delta H_s / RT^2) \quad (5)$$

Although it may not be absolutely correct to use K_m for calculation of ΔH_s , other authors have also used this approach to compare AspATs from different sources (see ref. [41]).

Thermal-stability measurements

The time-dependence of thermal inactivation of the halophilic AspAT was determined by heating enzyme solutions in sealed

tubes at different temperatures. After a specific heating time the samples were cooled on ice and assayed for remaining transaminase activity. The residual activity was determined by comparing the enzyme activity after heating with that of freshly prepared unheated enzyme solutions. The activation parameters for the thermal inactivation of the halophilic AspAT at these temperatures were calculated from the first-order inactivation plots. The inactivation rate constants were obtained from the slopes of the plots and were used to calculate the activation free energies of inactivation ($\Delta G^\ddagger$) at each temperature according to the following equation [23]:

$$\Delta G^\ddagger = -2.303RT[\log(k_i/T) - \log(R/Nh)] \quad (6)$$

where k_i is the first-order rate constant and N is Avogadro's number. The activation energy (E_a) for the thermal inactivation of the halophilic AspAT was obtained from the slope of the Arrhenius plot [$E_a = -R(\text{slope})$] [24]. Activation enthalpies ($\Delta H^\ddagger$) at each temperature were then calculated using the relationship [23]:

$$\Delta H^\ddagger = E_a - RT \quad (7)$$

and from the known values of $\Delta G^\ddagger$ and $\Delta H^\ddagger$, the activation entropies at each temperature were calculated from [23]:

$$\Delta S^\ddagger = (\Delta H^\ddagger - \Delta G^\ddagger)/T \quad (8)$$

The thermal stability of the halophilic AspAT in the presence of 2-oxoglutarate or L-aspartate and different concentrations of KCl was determined by heating the enzyme in the presence of each substrate or/and salt in sealed tubes for different times at 60 °C. Enzyme activity was measured before and after heating to determine residual activity under each condition.

RESULTS AND DISCUSSION

Calorimetric data

As shown in Figure 1, a single broad transition of the halophilic AspAT is observed over the range 3.3 to 1.75 M KCl. The values of the transition or denaturation temperature (T_d) ranged from 78.5 °C (3.3 M KCl) to 68.7 °C (1.75 M KCl), at pH 7.8. The calorimetric enthalpic changes ($\Delta_d H_{cal}$) associated with the conversion of the native form into the unfolded form of the halophilic AspAT were found to be 278.02 kJ·mol⁻¹ at 3.3 M KCl (physiological or nearly physiological conditions), 263.36 kJ·mol⁻¹

Figure 1 Excess of specific heat capacity of the halophilic AspAT as a function of temperature and salt concentration

Protein concentration was 0.8 mg/ml in 0.05 M sodium phosphate buffer, pH 7.8, containing KCl at the indicated molar concentrations (arrows).

Figure 2 Calorimetric enthalpy of the halophilic AspAT denaturation as a function of transition temperature

Arrows indicate the molar concentration of KCl. The value for the apparent molar heat capacity (C_p) was found to be 0.61, 0.72, 0.76 or 0.79 $\text{kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ at 1.75, 2.23, 2.63 or 3.30 M KCl (pH 7.8) respectively.

Figure 3 Dependence on temperature of the activity of the halophilic AspAT

The enzyme was assayed at different temperatures in 0.05 M sodium phosphate buffer, pH 7.8, containing KCl at the indicated molar concentrations. The activity observed at 65 °C in the presence of 3.5 M KCl is expressed as 100%.

at 2.63 M KCl, 246.61 $\text{kJ} \cdot \text{mol}^{-1}$ at 2.23 M KCl and 207.67 $\text{kJ} \cdot \text{mol}^{-1}$ at 1.75 M KCl. These values are lower than those observed in mammal AspATs [25]. In addition, Figure 2 displays a plot of calorimetrically obtained enthalpy values (ΔH_{cal}) of the halophilic AspAT denaturation versus transition temperature (T_d). This plot shows a straight line with a slope value of 9.88 $\text{kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$, which coincides with the change in apparent molar heat capacity on denaturation ($\Delta_a C_p$), in agreement with Kirchhoff's law:

$$\Delta C_p = \Delta H_{\text{cal}}/T \quad (9)$$

The C_p values obtained at each T_d are also described in the legend to Figure 2. These data on apparent molar heat capacity of the halophilic AspAT, which are proportional to non-polar contacts that stabilize the protein structure [26,27], are lower than those found for globular proteins. Therefore, it suggests that thermally induced order-disorder transitions of the halophilic AspAT are

accompanied by a reduction of hydrophobic interactions within the protein, just the type of interaction involved in the native halophilic transaminase and other native halophilic enzymes [10,11,28–31]. Thermal denaturation occurs by decreasing the activity coefficients of non-polar molecules [32] and the intensity of well-developed hydrophobic cores which are water-exposed at the transition temperature [33].

Effect of temperature on optimum activity

As shown in Figure 3, the optimum reaction temperature of the halophilic AspAT (at pH 7.8) was found to be 65 °C at 3.5, 2.4 or 1.6 M KCl, and 55 °C at 0.7 M KCl. Our results confirm a high optimum reaction temperature for the halophilic AspAT. Plotting the logarithm of the initial velocities between 35 and 65 °C against the reciprocal of the absolute temperature gave straight lines from which Arrhenius behaviour and thermodynamic activation parameters were obtained (Table 1). Like several other thermophilic and halophilic enzymes [34–37], AspAT from the halophile *Haloferax mediterranei* shows a thermal transition in conformation as indicated by the break in the Arrhenius plots around 48 °C. AspAT from the thermoacidophile *Sulfolobus solfataricus* also shows this behaviour [38]. As already demonstrated for aldolase and L-lactate dehydrogenase from thermophilic bacilli [36,39] this change was characterized by an abrupt change in the $\Delta H^*/\Delta S^*$ ratio, with a discontinuous increase of the entropic contribution to ΔG^* above this transition temperature (Table 1). Until now no structural details have been available to indicate the dimensions and localization of this conformational change, which may be a general consequence of the thermophilic structure responsible for maintaining both activity and stability at high temperatures and high ionic strengths.

The temperature decreased the value of the apparent K_m (10.20 ± 0.35 mM at 20 °C, 8.70 ± 0.22 mM at 30 °C, 6.95 ± 0.21 mM at 40 °C, 5.62 ± 0.26 mM at 50 °C and 4.55 ± 0.28 mM at 60 °C) for L-aspartate, whereas it increased the value of the apparent K_m (0.82 ± 0.01 mM at 20 °C, 0.85 ± 0.02 mM at 30 °C, 0.88 ± 0.01 mM at 40 °C, 0.92 ± 0.02 mM at 50 °C and 0.95 ± 0.02 mM at 60 °C) for 2-oxoglutarate. These results, analysed at 3.5 M KCl and pH 7.8 by a direct-linear-plot method of Eisenthal and Cornish-Bowden [40], are not consistent with the increased K_m concomitant with increases in temperature reported for mammal AspATs [41]. The variation of K_m with temperature was log-linear with respect to reciprocal of temperature (Figure 4). Apparent standard enthalpy changes (ΔH_s) for substrate binding were found to be -16.43 $\text{kJ} \cdot \text{mol}^{-1}$ for L-aspartate and 3.04 $\text{kJ} \cdot \text{mol}^{-1}$ for 2-oxoglutarate. The lower ΔH_s found for L-aspartate may be related to an apparent positive cooperativity under the described conditions [41] and may give a representative value for the heat of formation for the enzyme-substrate complex [21,42].

Effect of temperature on stability

The halophilic enzyme was found to be stable up to 50 °C for several hours (results not shown) under physiological or nearly physiological conditions (3.5 M KCl and pH 7.8). Similar results were found in the presence of L-aspartate. However, a shift of the thermal inactivation curves to higher temperatures was caused by 2-oxoglutarate. Inactivation followed first-order kinetics at all these temperatures and in the absence or presence of substrates, suggesting that, under the conditions used in these experiments, thermal unfolding of the halophilic AspAT was primarily responsible for the inactivation. The thermal stability of the

Table 1 Arrhenius behaviour and thermodynamic activation parameters for the thermal activation of the halophilic AspAT as a function of salt concentration

Some of the values for 3.5 M KCl were obtained from Muriana et al. [10].

Parameter	KCl concn. (M)			
	0.7	1.6	2.4	3.5
Arrhenius slope (K^{-1}) (between 35 and 45 °C)	−6313	−6274	−5882	−5882
Arrhenius slope (K^{-1}) (between 50 and 65 °C)	—	−1058	−1204	−1204
E_a ($kJ \cdot mol^{-1}$) (between 35 and 45 °C)	58.83	54.97	48.91	48.91
E_a ($kJ \cdot mol^{-1}$) (between 50 and 65 °C)	—	8.82	9.99	9.99
$\Delta H^*_{37^\circ C}$ ($kJ \cdot mol^{-1}$)	56.22	52.42	46.33	46.33
$\Delta H^*_{40^\circ C}$ ($kJ \cdot mol^{-1}$)	56.20	52.39	46.30	46.30
$\Delta H^*_{45^\circ C}$ ($kJ \cdot mol^{-1}$)	56.16	52.36	46.26	46.26
$\Delta H^*_{50^\circ C}$ ($kJ \cdot mol^{-1}$)	—	6.11	7.32	7.32
$\Delta H^*_{55^\circ C}$ ($kJ \cdot mol^{-1}$)	—	6.07	7.27	7.27
$\Delta H^*_{60^\circ C}$ ($kJ \cdot mol^{-1}$)	—	6.03	7.23	7.23
$\Delta H^*_{65^\circ C}$ ($kJ \cdot mol^{-1}$)	—	5.99	7.19	7.19
$\Delta G^*_{37^\circ C}$ ($kJ \cdot mol^{-1}$)	58.37	58.13	57.97	57.73
$\Delta G^*_{40^\circ C}$ ($kJ \cdot mol^{-1}$)	58.96	58.72	58.55	58.31
$\Delta G^*_{45^\circ C}$ ($kJ \cdot mol^{-1}$)	59.95	59.69	59.53	59.28
$\Delta G^*_{50^\circ C}$ ($kJ \cdot mol^{-1}$)	—	60.67	60.51	60.26
$\Delta G^*_{55^\circ C}$ ($kJ \cdot mol^{-1}$)	—	61.66	61.49	61.23
$\Delta G^*_{60^\circ C}$ ($kJ \cdot mol^{-1}$)	—	62.64	62.47	62.21
$\Delta G^*_{65^\circ C}$ ($kJ \cdot mol^{-1}$)	—	63.62	63.45	63.18
$\Delta S^*_{37^\circ C}$ ($J \cdot mol^{-1} \cdot K^{-1}$)	−6.93	−18.42	−37.55	−36.77
$\Delta S^*_{40^\circ C}$ ($J \cdot mol^{-1} \cdot K^{-1}$)	−8.82	−20.22	−39.14	−38.37
$\Delta S^*_{45^\circ C}$ ($J \cdot mol^{-1} \cdot K^{-1}$)	−11.92	−23.05	−41.73	−40.94
$\Delta S^*_{50^\circ C}$ ($J \cdot mol^{-1} \cdot K^{-1}$)	—	−168.92	−164.68	−163.90
$\Delta S^*_{55^\circ C}$ ($J \cdot mol^{-1} \cdot K^{-1}$)	—	−169.48	−165.30	−164.51
$\Delta S^*_{60^\circ C}$ ($J \cdot mol^{-1} \cdot K^{-1}$)	—	−170.00	−165.89	−165.10
$\Delta S^*_{65^\circ C}$ ($J \cdot mol^{-1} \cdot K^{-1}$)	—	−170.50	−166.45	−165.65

Figure 4 Dependence on temperature of $\ln K_m$ of the halophilic AspAT

Napierian logarithms of K_m (mM) of the enzyme for L-aspartate (○) and 2-oxoglutarate (●) are shown. Concentrations of non-varied substrates were 10 mM (2-oxoglutarate) and 100 mM (L-aspartate) in 0.05 M sodium phosphate buffer, pH 7.8, containing 3.5 M KCl.

halophilic AspAT was significantly greater in the presence of 2-oxoglutarate than that of most well-studied transaminases from mesophiles and was similar to the remarkable resistant to heat-inactivation of the truly thermophilic transaminase from *S. solfataricus* [38]. The slope of the Arrhenius plots, constructed from the first-order rate constants at each temperature, cor-

responded to activation energies for the thermal inactivation of 200.60 $kJ \cdot mol^{-1}$ in the absence of substrates, 206.30 $kJ \cdot mol^{-1}$ in the presence of L-aspartate and 238.86 $kJ \cdot mol^{-1}$ in the presence of 2-oxoglutarate, reflecting the high thermal stability of the halophilic transaminase. Other calculated activation parameters for thermal inactivation of the halophilic AspAT are listed in Table 2. Over the temperature range between 60 and 85 °C, neither $\Delta H^{\ddagger}$ nor $\Delta S^{\ddagger}$ was significantly affected by temperature, whereas $\Delta G^{\ddagger}$ decreased linearly with temperature. Voordouw et al. [43] have proposed that kinetic thermal stability should be used for defining thermostable enzymes. In addition, these authors emphasize that the resistance of enzymes to thermal denaturation is due to the 'intrinsic' contribution of the polypeptide chain (i.e. hydrophobic interactions, hydrogen-bonding and ionic stabilization) and an enzyme with a $\Delta G^{\ddagger} \geq 104.6$ $kJ \cdot mol^{-1}$, at 70 °C and under optimal conditions for activity, may be considered as a thermostable enzyme. As shown in Table 2, the values of $\Delta G^{\ddagger}$ for the stability of the halophilic AspAT at 70 °C were found to be 105.84 $kJ \cdot mol^{-1}$, 105.93 $kJ \cdot mol^{-1}$ and 112.17 $kJ \cdot mol^{-1}$, in the absence of substrates or in the presence of L-aspartate or 2-oxoglutarate respectively. Therefore, according to the above definition, the halophilic AspAT may just be qualified as a thermostable enzyme, even in the absence of substrates.

To assess the contribution of ionic strength to thermal stabilization of the halophilic AspAT, the effect of KCl at different concentrations on the kinetic thermal stability of the enzyme was studied at 60 °C. As expected, the thermal stability of the halophilic AspAT was highly dependent on salt concentration

Table 2 Thermodynamic activation parameters for the thermal inactivation of the halophilic AspAT as a function of temperature at 3.5 M KCl

T(°C)	$\Delta G^\ddagger$ (kJ·mol ⁻¹)	$\Delta H^\ddagger$ (kJ·mol ⁻¹)	$\Delta S^\ddagger$ (kJ·mol ⁻¹ ·K ⁻¹)
No substrate			
60	108.66	197.83	0.27
65	106.96	197.79	0.27
70	105.84	197.75	0.27
75	104.54	197.71	0.27
80	103.15	197.67	0.27
85	101.61	197.63	0.27
L-Aspartate			
60	109.13	203.53	0.28
65	107.14	203.49	0.28
70	105.93	203.45	0.28
75	104.59	203.41	0.28
80	103.06	203.37	0.28
85	101.65	203.32	0.28
2-Oxoglutarate			
60	115.34	236.09	0.36
65	113.77	236.05	0.36
70	112.17	236.01	0.36
75	109.83	235.97	0.36
80	107.92	235.92	0.36
85	106.38	235.88	0.36

Figure 6 Relationship between the relative change in free energy of activation $\Delta(\Delta G^\ddagger)$ for the thermal inactivation of the halophilic AspAT at 60 °C and the concentration of KCl at fixed molal surface tension

(Figures 5a and 5b). The halophilic AspAT was more thermostable at a high KCl concentration (3.5 M KCl and pH 7.8). An estimate of the stabilizing/destabilizing effect of the KCl may be obtained from the difference in activation free energies of thermal inactivation at low or high salt concentration, according to the expression

$$\Delta(\Delta G^\ddagger) = \Delta G_L^\ddagger - \Delta G_H^\ddagger \quad (10)$$

where $\Delta G_L^\ddagger$ and $\Delta G_H^\ddagger$ are the activation free energies at low or high salt concentrations respectively, calculated according to eqn. (6) above. The calculated values of $\Delta(\Delta G^\ddagger)$, with respect to $\Delta G_L^\ddagger$ at 0.97 M KCl, for the halophilic AspAT are described in Figure 6. The thermal stability of the halophilic AspAT was correlated with salt concentration. The mechanism by which KCl, as a lyotropic salt, affects halophilic protein stability may be explained by the effect of such salts on water structure and hence on the strength of hydrophobic interactions. Kristjansson and Kinsella [44], in accordance with the theory of the salt effects on protein hydrophobic interactions from Melander and Horvath [32], found that the effect of lyotropic salts on hydrophobic interactions in a thermostable enzyme may be described by their effect on the surface tension of water, which is quantified by their molal surface increment. The correlation between hydrophobic interactions and the surface tension of water is expected if hydrophobicity arises from the energy required to make a cavity for exposed non-polar groups of proteins in water [45]. The free energy associated with the formation of such a cavity in the solvent ($\Delta G_{\text{cav.}}$) can be expressed by the relationship [32]

$$\Delta G_{\text{cav.}} = [NA + 4.8N^{\frac{1}{3}}(k^e - 1)V^{\frac{2}{3}}]\nu^0 + [NA + 4.8N^{\frac{1}{3}}(K^e - 1)V^{\frac{2}{3}}]\sigma m \quad (11)$$

where N is Avogadro's number, A is the molecular surface area of the protein molecule, V is the molar volume of the solvent, ν^0 is the surface tension of pure water, m is the molality of the salt and σ is the molal surface tension increment and is a constant for a given salt (the value for σ of KCl was found to be 1.49×10^{-2} N·g·cm⁻¹·mol⁻¹ [32]). k^e corrects the macroscopic surface tension of the solvent to molecular dimensions [32]. Assuming that A , k^e and V are unaffected by the presence of salt, it can be expected, from eqn. (11), that $\Delta G_{\text{cav.}}$ should be directly proportional to σm , the molal surface tension increment and the molality of the salt. On the other hand, as hydrophobicity arises from the free energy required to form a cavity for the non-polar groups in the solvent, and the salt is affecting the hydrophobic

Figure 5 (a) First-order plots for the thermal inactivation of the halophilic AspAT at 60 °C and as a function of salt concentration and (b) dependence on salt concentration of $\ln k_i$

(a) The enzyme was incubated at 60 °C in 0.05 M sodium phosphate buffer, pH 7.8, containing KCl at the indicated molar concentrations. Aliquots were withdrawn to determine the remaining activity expressed relative to that of an unheated sample. (b) Napierian logarithms of the inactivation rate constants obtained from (a) are shown.

interactions of the protein and σ is a constant for a given salt, the observed stabilization effect on the halophilic AspAT should be directly proportional just to the salt concentration. Indeed, a plot of $\Delta(\Delta G^\ddagger)$ in the presence of different KCl concentrations gave a strong linear relationship (Figure 6). This result indicates that the primary effect of KCl on the thermal stability of the halophilic AspAT can be ascribed to the effect on hydrophobic interactions in a salt-concentration-dependent manner.

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