Stability of aspartate aminotransferase from *Sulfolobus solfataricus*

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Aspartate aminotransferase from *Sulfolobus solfataricus* (SsAspAT) is an extremely thermophilic and thermostable dimeric enzyme which retains its structure and reaches maximal activity at 100°C. The structural stability of this protein was investigated by coupling isothermally and thermally induced denaturation studies to molecular modeling. Gel filtration analysis indicated that SsAspAT unfolds with an $N_2 = 2D$ mechanism. In the molecular model, a cluster of hydrophobic residues was shown at the interface between the subunits of SsAspAT and suggested this cluster as a structural feature stabilizing the enzyme quaternary structure. At 25°C, SsAspAT is less resistant to guanidinium chloride-induced denaturation than the cytosolic aspartate aminotransferase from pig heart (cpAspAT), which was chosen as a mesophilic counterpart in the thermodynamic analysis since it shares with SsAspAT the two-state unfolding mechanism. Therefore, in the case of aspartate aminotransferases, thermal stability does not correlate with the stability against chemical denaturants. Isothermal denaturation curves at 25°C and melting profiles recorded in the presence of guanidinium chloride showed that the $\Delta G°(H_2O)$ at 25°C of SsAspAT exceeds that of cpAspAT by roughly 15 kJ/mol; the parameter $\Delta_n$, related to the number of binding sites for the denaturant differentially exposed in unfolded and folded states, is higher for SsAspAT than for cpAspAT; and $\Delta C_p$ is lower for the thermophilic enzyme than for the mesophilic one by 8 kJ/K.mol. These results are indicative of a less hydrophobic core for SsAspAT than cpAspAT. In agreement with this, the molecular model predicts that some charged side chains are buried in SsAspAT and interact to form an H-bond/ion-pair network.

**Keywords**: aspartate aminotransferase/chemical unfolding/dimeric protein/molecular modeling/thermostability

**Introduction**

Despite the great deal of work carried out in recent years, the molecular mechanism by which protein structure adapts to extreme physical conditions is still not completely understood.

In addition to studies based on approaching the protein stability problem by either studying the thermodynamics of protein unfolding (Makahata and Privalov, 1993) or evaluating the effect of selected changes that were introduced in the protein architecture by site-directed mutagenesis, the comparison of proteins performing similar functions in organisms living at different temperatures has also been instrumental in evaluating how external environmental factors, such as temperature, force the molecular adaptation of proteins towards greater stability and in understanding which forces have been naturally selected in order to stabilize the native conformation of globular proteins.

This comparative analysis suggests mutative adaptation of proteins to specific physiological conditions to maintain ‘corresponding states’ regarding structure, flexibility, ligand binding and catalysis (Jaenicke, 1991), while the examination of the factors effecting the enhanced stability of thermophilic proteins indicates no single influence as producing the major effect with individual proteins exhibiting different combinations of stabilizing effects (Menéndez-Arias and Argos, 1989).

In this paper, we report an analysis of the stability of aspartate aminotransferase isolated from *Sulfolobus solfataricus* (SsAspAT), a sulphur-dependent archaeon, that is an enzyme stable and fully active at 100°C (Marino et al., 1988). This protein was chosen because its mesophilic counterparts are well characterized and because of the reversibility of the unfolding process.

Aspartate aminotransferase from *S.solfataricus* shares with its mesophilic eukaryotic and bacterial counterparts a large number of catalytically and structurally significant features, but only a low percentage of identical amino acids, not exceeding 20% (Cubellis et al., 1989). It is worth noting, however, that the two bacterial thermophilic aspartate aminotransferases that have been sequenced so far, namely AspAT from *Bacillus* species (Sung et al., 1991) and *Thermus thermophilus* (Okamoto et al., 1996), also show a low percentage of similarity with the mesophilic aspartate aminotransferases but are much more similar to the archaeal thermophilic enzyme. The differences observed, therefore, which may also reflect the evolutionary distance between Archaea, Eukarya and Bacteria, can mainly account for the enzyme adaptation to high temperatures.

In this paper, guanidinium chloride (GuHCl) and thermally induced unfolding of SsAspAT are presented and discussed in relation to a molecular model of the enzyme in an attempt to define the molecular basis of stability of this extremely thermophilic protein.

**Materials and methods**

*Protein purification and enzymatic assays*

cpAspAT was purified from pig heart as described by Porter et al. (1981) while recombinant SsAspAT was expressed in *E.coli* and purified as described by Arnone et al. (1992a). Protein concentration was determined spectrophotometrically from the absorption coefficient at 278 nm ($A_278 = 1.48$ for cpAspAT (Bergami et al., 1968) and 1.3 for SsAspAT). The molar concentration per subunit was calculated using

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*This paper is dedicated to the memory of our much missed friend and colleague Gianpaolo Nitti, who first started working on *Sulfolobus solfataricus* aspartate aminotransferase in the Naples laboratory.*

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enzymes (maximum 1/20 of the final volume) to a reaction. Fluorescence spectra were corrected by the small apparent absorbance due to the enzyme at 412 nm due to the addition of the enzymes (maximum 1/20 of the final volume) to a reaction mixture containing 2.0 mM 2-oxoglutarate and 13.0 mM l-cysteine sulfinate in 50 mM Tris–HCl (pH 8.5), 0.1 mM EDTA and 0.15 mM 5,5′-dithiobis(2-nitrobenzoic acid), following the method described by Marino et al. (1988) for SsAspAT. When the enzymatic activity of samples preincubated in the presence of GuHCl was measured, the final concentration of GuHCl in the reaction mixture never exceeded 30 mM. Moreover, in all cases the progression curves of absorbance versus time were linear for at least 1 min, thus indicating that the measurements had been carried out fast enough to prevent denaturation during the assays that would have led to a concave-up progress curve.

Guanylguanidinium chloride-induced unfolding

GuHCl of spectroscopic grade was purchased from BDH or Pierce. SsAspAT or cpAspAT (0.17 mg/ml) were incubated in 10 mM Na-MOPS (pH 7.5) in the presence of various concentrations of GuHCl at 25°C until the unfolding equilibrium was attained (60–180 min). The concentration of GuHCl was determined refractometrically using tabulated values of the solution refractive index (Pace, 1986). The activity of GuHCl was determined spectrophotometrically using the values determined by Makhatadze and Privalov (1992) for solutions with given molarities at 25°C.

In the isothermal GuHCl-induced unfolding experiments, the extent of unfolding was determined for each solution by measuring the residual enzyme activity, molar ellipticity at 220 nm and the shift of maximum fluorescence emission (after excitation at 280 or 295 nm). The progress of thermally induced unfolding was followed by measuring the change in molar ellipticity at 220 nm.

Renaturation of unfolded enzymes was carried out in 0.1 M Na-MOPS (pH 7.5), 0.1 mM PMP, 2 mM 2-oxoglutarate and in 0.1 M Na-MOPS (pH 7.5), 5 mM dithiothreitol, 0.1 mM PLP for SsAspAT and cpAspAT, respectively.

Spectral measurements

All experiments were carried out on 0.17 mg/ml solutions of SsAspAT or cpAspAT.

Far-UV CD measurements were performed on cells of 1 mm path length, using a JASCO J-300 A automatic recording spectropolarimeter equipped with a Lauda RCS thermostat. The temperature was monitored by means of a laboratory-made thermistor sensor which penetrated through a silicone-rubber cap into the sample cell. Spectra were recorded in the range 210–250 nm. The ellipticity at 220 nm of samples in the presence of various concentrations of GuHCl at 25°C was measured. Melting profiles were recorded by raising the temperature (~2°C/min) of samples equilibrated at different concentrations of GuHCl. The molar ellipticities were calculated from the observed ellipticity values, θ_{obs}, according to

\[ [\theta]_{220} = \frac{M_w \times \theta_{obs}}{10 \times l \times c} \]

where \( M_w \) is the molecular weight of monomers, 45 000 and 46 000 Da for SsAspAT and cpAspAT respectively, \( l \) is the path length of the cell (0.1 cm) and \( c \) is the protein concentration expressed in mg/ml.

Fluorescence spectra were recorded on a Perkin-Elmer MPF66B spectrophotometer. Sample temperature was determined with a thermocouple inserted into the cuvette. The fluorescence spectra were corrected by the small apparent fluorescence of the solutions of GuHCl. The shift of maximum fluorescence emission upon denaturation was monitored (excitation at 295 or 280 nm, 5 nm bandwidths on both excitation and emission sides).

Samples were equilibrated in GuHCl before any spectroscopic measurements for 60–180 min.

Gel filtration analysis

Gel filtration analysis of SsAspAT and cpAspAT was carried out on a Superose 12 PC (3.2×300 mm) column using the Smart System (Pharmacia LKB). The column was calibrated with the following proteins of known molecular weight: β-amylase (200 000 Da), cpAspAT (92 000 Da), bovine hemoglobin (67 000 Da), cytochrome c (12 300 Da). Volumes of 50 μl of cpAspAT and SsAspAT (0.17 mg/ml) were equilibrated, loaded on the column and isocratically eluted with 50 mM MOPS (pH 7.5), 0.15 M NaCl at various concentrations of GuHCl. All experiments were carried out at 25°C at a flow rate of 40 μl/min.

Data analysis

Data analysis and resolution of numerical equations were performed using the computer program Regression (Blackwell Scientific Software, Oxford, UK).

Molecular modeling

Homology modeling of SsAspAT was carried out with the modules HOMOLOGY and DISCOVER in the package INSIGHT II (Biosym Technologies). The model was optimized and refined with several cycles of energy minimization using both the conjugate gradient and the steepest descent minimizers with the consistent valence force field. The quality and the stereorechemical properties of the model were assessed with the method of Lüthy et al.(1992) and the program PROCHECK (Laskowsky et al., 1993), respectively. Secondary structure assignments and accessibility calculations were carried out with the program DSSP (Kabsch and Sander, 1983). Figures showing molecular structures were produced with the program MOLSCRIPT (Kraulis, 1991). Analysis of potential hydrogen bonds was carried out with HBPLUS (McDonald and Thornton, 1994). Modeling and structure analysis were carried out on Indigo and Indigo2 stations. The sequence numbering system adopted throughout the paper refers to the positions in the cpAspAT sequence (see Figure 6).

Results

GuHCl-induced unfolding: setting up of experimental conditions

Unfolding of SsAspAT at 25°C was induced by addition of GuHCl, by incubating samples of the enzyme (0.17 mg/ml) in 10 mM Na-MOPS (pH 7.5) at different concentrations of the denaturant. After 1 h of incubation, the samples were assayed under standard conditions for enzymatic activity every 30 min for at least 4 h. Equilibrium was attained within the first hour of incubation since residual enzymatic activity was found to depend on GuHCl concentration, and not on the incubation time. Complete denaturation of SsAspAT could be obtained...
Unfolding and molecular modeling analysis of \( Ss \) AspAT

Fig. 2. Effect of temperature on the reactivation of aspartate aminotransferase from \( S. solfataricus \). The enzymes (0.17 mg/ml) were incubated at 25°C for 1 h in 50 mM Na-MOPS (pH 7.5), 0.15 M NaCl at 4 M GuHCl and then diluted 20-fold in 0.1 M MOPS (pH 7.5), 0.1 mM PMP, 2 mM 2-oxoglutarate. Standard assays at 60°C were performed. Temperature dependence at 25°C (▲), 37°C (●) and 50°C (■). Relative activity refers to final yields after 24 h.

To test whether unfolding of \( Ss \) AspAT is a reversible process, the kinetics of the reactivation process were measured at different temperatures after denaturation in 4 M GuHCl at 25°C. A complex temperature dependence of reactivation was observed (Figure 2), similarly to other thermophilic enzymes (Jaenicke, 1996). In fact, refolding of \( Ss \) AspAT can be accelerated by increasing the temperature from 25 to 50°C. However, although refolding is faster at 50°C (35% of native enzyme), the highest yield of renaturation is obtained at 37°C (70% reactivation), similar to the yield of 65% of initial activity that is obtained at 25°C.

Since AspATs are homodimeric enzymes, the overall unfolding reaction must start from a folded dimer \((N_2)\) and end up with two unfolded monomers \((2D)\). However, which unfolding pathway is actually followed among several possibilities is dependent on the relative stability of the intermediates and the experimental conditions. For instance, gel filtration analysis has shown that a stable structured monomeric species is an intermediate on the complicated unfolding pathway of aspartate aminotransferase from \( E. coli \) (Herold and Kirschner, 1990; Herold and Leistler, 1991). To test whether this possibility applies to \( Ss \) AspAT too, protein samples were incubated (under the conditions described above) in the presence of 4.0 and 6.0 M GuHCl for 1–2 h to attain unfolding equilibrium and were then analyzed by gel filtration. Equilibration of the column and elution were carried out at the same concentration of GuHCl as used in the denaturing incubation of the samples. As shown in Figure 3A and B, native and denatured proteins do not exchange rapidly since two peaks were revealed in the case of \( Ss \) AspAT at 2.7 and 3.0 M GuHCl. No species with

Fig. 1. GuHCl-induced denaturation curves at 25°C of aspartate aminotransferases from \( S. solfataricus \) (\( Ss \) AspAT) (A) and from pig heart (cpAspAT) (B). Apparent fractions of native protein, \( F_{app} \), were derived from the experimental data by Equation 1, assuming a two-state transition of unfolding. Changes in molar ellipticity at 220 nm (▲) or the shift of fluorescence emission peak (●) were used as different probes to monitor unfolding. Fluorescence data are upon excitation at 295 nm for \( Ss \) AspAT and at 280 nm for cpAspAT. Denaturation was carried out at 25°C in 10 mM Na-MOPS (pH 7.5) at the indicated concentrations of GuHCl for 1 h. Residual enzymatic activity (○), measured under standard conditions, is also reported as a function of the GuHCl in the denaturing incubation.

To test whether GuHCl-induced denaturation of \( Ss \) AspAT is accompanied by complete unfolding, fluorescence spectra of the enzyme were recorded in the presence of different concentrations of GuHCl. Denaturation in 6.0 M GuHCl caused a bathochromic shift of the emission peak (excitation at 295 nm) from 315 to 348 nm with a significant increase in fluorescence intensity (data not shown). Emission spectra of \( Ss \) AspAT incubated in 4.0 and 6.0 M GuHCl are superimposable. The CD spectra confirm that the optical properties of \( Ss \) AspAT in 4.0 and 6.0 M GuHCl are very similar (data not shown). The activity, fluorescence and circular dichroism measurements indicate that functional structure as well as tertiary and secondary structures are lost in solutions of GuHCl more concentrated than 3.5 M and, therefore, \( Ss \) AspAT was considered to be fully unfolded in the presence of denaturant concentrations >3.5 M.

A single transition is observed (Figure 1A), with no spectrscopically detectable intermediate, and isothermal denaturation curves obtained for \( Ss \) AspAT using different spectroscopic approaches are superimposable.
Fig. 3. Gel filtration chromatography of aspartate aminotransferases from *S. solfataricus* (*Ss*AspAT) (A and B), and from pig heart (cpAspAT) (C and D) in the presence of GuHCl. The enzymes (0.17 mg/ml) were incubated at 25°C for 1 h in 50 mM Na-MOPS (pH 7.5), 0.15 M NaCl at the indicated GuHCl concentrations. Samples (50 µl) were loaded on to the column previously equilibrated in the same buffered solution of GuHCl and isocratically eluted at a flow rate of 40 µl/min. Some representative elution profiles are shown in (A) (*Ss*AspAT) and (C) (cpAspAT), and the elution volumes of native (●) and denatured (■) states in (B) (*Ss*AspAT) and (D) (cpAspAT). Under the same conditions the elution volume of AspAT from *E. coli* was shifted from 1.26 ml in the native state to 1.33 ml, corresponding to the monomeric intermediates, for concentrations of GuHCl in the range 0.4–1 M, and then to 1.09 ml in the unfolded state at 4 M GuHCl (data not shown).

Stokes radii smaller than those of native homodimers is observed at any guanidinium chloride concentration. Therefore, it can be suggested that, at least under experimental conditions tested, unfolding of *Ss*AspAT does not proceed through the formation of stable structured monomers as is the case of the *E. coli* enzyme. The data presented in Figure 3 refer to samples isocratically eluted from a gel filtration column after conformational equilibrium in the presence of a specific concentration of GuHCl had been attained. Further evidence against the occurrence of a dissociation step to stable compact intermediates was obtained using the method described by Endo et al. (1983). In fact, species with Stokes radii smaller than that of native homodimer were not detected even when samples of native *Ss*AspAT were repeatedly injected and analyzed by gel filtration along a gradient of increasing GuHCl concentration (data not shown). These experiments were on a time-scale of analysis ranging from 20 to 40 min, considerably shorter than in the experiments at the equilibrium (1 h equilibration plus 20–40 min for the gel filtration analysis).

Thus, activity and spectroscopic analysis combined with gel filtration experiments allowed us to consider *Ss*AspAT unfolding as a two-state N → 2D process. Therefore, in a comparative analysis of the thermodynamic properties of the thermophilic AspAT, *Ec*AspAT, the aspartate aminotransferase that shares with *Ss*AspAT the highest sequence similarity, has to be excluded as the mesophilic counterpart, since its unfolding process proceeds through two monomeric intermediates (Herold and Kirschner, 1990).

Similar preliminary unfolding experiments have shown that porcine cytosolic aspartate aminotransferase (cpAspAT) is a suitable mesophilic counterpart in a thermodynamic analysis of *Ss*AspAT. In fact, under the same experimental conditions as tested for *Ss*AspAT, GuHCl-induced unfolding of cpAspAT is reversible and can be considered as a two-state N → 2D process, since no activity or spectroscopically detectable intermediates can be observed, isothermal denaturation curves obtained for cpAspAT using different approaches are superimposable (Figure 1B) and no species with Stokes radii smaller
Fig. 4. Plot of free energy of unfolding $\Delta G^\circ$ at 25°C versus $\ln(1 + ka)$, where $a$ is the activity of guanidinium chloride and $k$ is the denaturant binding constant. Data referring to SsAspAT are indicated by open symbols and those referring to cpAspAT by closed symbols. $\Delta G^\circ$ values were calculated from circular dichroism ($\Delta \varepsilon_0$, $\Delta \varepsilon$), fluorescence (☐, □) and residual enzymatic activity (●, ○) measurements. $\Delta G^\circ$ values were calculated from $F_{app}$ values in the transition region of Figure 1 using Equation 2. A $k$ value of 0.6 was used (Makhatadze and Privalov, 1992). The activity of GuHCl was determined analytically using the values determined by Makhatadze and Privalov (1992) for solutions with given molarities at 25°C.

Unfolding of cpAspAT was induced by incubating the enzyme (0.17 mg/ml) at different concentrations of GuHCl at 25°C. Equilibrium was attained within the first hour of incubation, since residual enzymatic activity was found to depend only on GuHCl concentration, and not on the incubation time even when incubation at 25°C was prolonged for over 4 h. Upon incubation in 6.0 M GuHCl, the fluorescence emission maximum (excitation at 280 nm) of cpAspAT was shifted from 333 to 348 nm and the protein was completely inactive and unfolded, as shown by enzymatic assays, fluorescence spectra and circular dichroism. GuHCl-induced unfolding of cpAspAT is a reversible process since removal of the denaturant by dialysis against at least 50 volumes of 0.10 M MOPS (pH 7.5), 5.0 mM diithiothreitol, 0.10 mM PLP at 4°C led to a recovery of 70% of the initial activity.

Isothermal denaturation at 25°C

Isothermal GuHCl-induced denaturation of SsAspAT at 25°C was analyzed with excitation at 295 nm and following the shift of the maximum of fluorescence emission as a measure of progressive unfolding. A second independent index of SsAspAT unfolding was also evaluated by measuring the molar ellipticity at 220 nm. In both cases, the apparent fraction of protein molecules in the native state ($F_{app}$) was calculated at different concentrations of GuHCl (Figure 1), using the equation

$$F_{app} = \frac{y - y_D}{y_N - y_D}$$

where $y$ is the shift in the fluorescence maximum or the molar ellipticity and $y_N$ and $y_D$ are the values of $y$ for the native and the denatured states, respectively. Figure 1A also shows the values of the residual enzymatic activity of SsAspAT incubated in the presence of GuHCl and assayed under standard conditions.

Similar experiments were carried out to analyze the isothermal GuHCl-induced denaturation of cpAspAT at 25°C. In this case, however, the excitation wavelength in the fluorescence experiments was 280 nm. Isothermal denaturation curves obtained for cpAspAT from activity, CD and fluorescence measurements are shown in Figure 1B.

The observations that isothermal denaturation curves obtained using different approaches for SsAspAT as well as for cpAspAT are superimposable and that no stable intermediates were found led to the assumption that, in both cases, the unfolding process can be approximated and treated as a two-state mechanism, $N_2 \rightleftharpoons 2D$. Relying on this assumption, equilibrium constants and $\Delta G^\circ$ for the process were calculated at each concentration of GuHCl from the $F_{app}$ values using the equation

$$K = \frac{[D]^2}{[N_2]} = 2P \frac{(1 - F_{app})^2}{F_{app}} = \exp(-\Delta G^\circ/RT)$$

where $P$ represents the total enzyme concentration as a monomer. Equilibrium constants, $K$, were also calculated setting the enzymatic activity of the native enzymes to 1 and substituting the relative enzymatic activity values to $F_{app}$ in Equation 2.

According to the binding model proposed by Tanford (1970) and validated by Makhatadze and Privalov (1992), the effect of guanidinium chloride can be understood in terms of the differential solvation of more groups in the unfolded state than in the native state. Thus GuHCl shifts the free energy gap between native and unfolded conformation so that at high enough concentration, the unfolded conformation is more stable than the native. This effect of GuHCl on $\Delta G^\circ$ of unfolding can be described by the equation

$$\Delta G^\circ = \Delta G^\circ(H_2O) - \Delta nRT\ln(1 + ka)$$

where $a$ represents the activity of GuHCl, $n$ is proportional to the number of residues differentially exposed to denaturant and $k$ is the binding constant.

$\Delta G^\circ(H_2O)$ and $\Delta n$ at 25°C for SsAspAT and cpAspAT were derived by plotting $\Delta G^\circ$ values measured in the presence of GuHCl as a function of $\ln(1 + ka)$ (Figure 4). A $k$ value of 0.60 reported by Makhatadze and Privalov (1992) was used.

Table I. Thermodynamic parameters characterizing the isothermal denaturation of AspATs in GuHCl at 25°C

<table>
<thead>
<tr>
<th></th>
<th>SsAspAT</th>
<th>cpAspAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta G^\circ(H_2O)$ (kJ/mol)</td>
<td>$\Delta n$</td>
<td>$\Delta G^\circ(H_2O)$ (kJ/mol)</td>
</tr>
<tr>
<td>Circular dichroism</td>
<td>76.4±4.9</td>
<td>30.4±4.2</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>65.9±5.2</td>
<td>24.3±5.0</td>
</tr>
<tr>
<td>Activity</td>
<td>67.5±5.5</td>
<td>27.1±5.4</td>
</tr>
<tr>
<td>Average value</td>
<td>70.3±5.2</td>
<td>27.7±4.8</td>
</tr>
</tbody>
</table>

$^a\Delta G^\circ$ values were calculated by fitting the data in the transition region of isothermal denaturation curves (Figure 1) to Equation 2. $\Delta G^\circ(H_2O)$ and $\Delta n$ were evaluated by nonlinear regression of $\Delta G^\circ$ values to Equation 3.

$^b$Value calculated as average of the values determined with the three different methods.
Table II. Thermodynamic parameters characterizing the thermal denaturation of AspATs at different GuHCl concentrations

<table>
<thead>
<tr>
<th>[GuHCl] (M)</th>
<th>$T_d$ ($^\circ$C)</th>
<th>$\Delta H$ (kJ/mol)</th>
<th>$\Delta S$ (kJ/mol.K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpAspAT</td>
<td>1.4</td>
<td>73.1</td>
<td>2.0 ± 0.10</td>
</tr>
<tr>
<td>SsAspAT</td>
<td>1.6</td>
<td>72.2</td>
<td>1.83 ± 0.08</td>
</tr>
<tr>
<td>SsAspAT</td>
<td>1.8</td>
<td>79.4</td>
<td>1.3 ± 0.04</td>
</tr>
<tr>
<td>cpAspAT</td>
<td>2.0</td>
<td>71.0</td>
<td>1.26 ± 0.07</td>
</tr>
<tr>
<td>SsAspAT</td>
<td>2.4</td>
<td>68.0</td>
<td>1.49 ± 0.05</td>
</tr>
<tr>
<td>SsAspAT</td>
<td>3.3</td>
<td>70.0</td>
<td>1.05 ± 0.04</td>
</tr>
</tbody>
</table>

*Protein concentration was 0.17 mg/ml and buffer was 10 mM Na-MOPS (pH 7.5).

$^b$Denaturation was monitored following the temperature-induced changes in ellipticity at 220 nm as described in the text. $T_d$ was estimated as the temperature at the midpoint of the thermal transition.

$^c$$\Delta G^\circ$ values were calculated in transition region for temperatures close to $T_d$ using Equation 2, and were then least squares fitted to the equation $\Delta G^\circ = \Delta H - T \Delta S$.

Fig. 5. Melting profiles of SsAspAT (circles) and cpAspAT (squares) in the presence of 2 M GuHCl. Open symbols indicate the ellipticity recorded on heating protein samples (0.17 mg/ml) previously equilibrated at 25 °C in 2 M GuHCl, 10 mM Na-MOPS (pH 7.5). Closed symbols indicate the ellipticity recorded on cooling samples previously heated to 76 °C.

The values calculated for SsAspAT and cpAspAT are reported in Table I. It is worth noting the rather good agreement among the values determined with different approaches leading to the average values of $\Delta G^\circ$ (H$_2$O) $= 70.3±5.2$ kJ/mol and $\Delta n = 27.7±4.8$ for SsAspAT and $\Delta G^\circ$ (H$_2$O) $= 57.6±1.7$ kJ/mol and $\Delta n = 12.0±0.9$ for cpAspAT.

**Thermal denaturation**

Thermal denaturation curves were obtained for SsAspAT and cpAspAT in the presence of different concentrations of GuHCl following the change in molar ellipticity at 220 nm as a measure of the progress of unfolding. Figure 5 shows the results of typical experiments carried out at 2 M GuHCl. As shown, thermally induced denaturation of both SsAspAT and cpAspAT can be considered a reversible process since almost superimposable values of ellipticity are obtained on heating and cooling the samples in the temperature range of the denaturation transition.

The concentrations of GuHCl were chosen on the basis of the observation that SsAspAT and cpAspAT are partially unfolded even at room temperature when denaturant is more concentrated than 2.4 and 2.6 M respectively (Figure 1A and B). $F_{app}$ was calculated using Equation 1, where $y_N$ and $y_D$ are the values of the molar ellipticity extrapolated from the native and the unfolded regions of the melting profiles. Apparent equilibrium constants $K_{app}$ and standard free energy changes $\Delta G^\circ$ at any temperature $T$ within the transition zone of the thermally-induced unfolding were determined using Equation 2. The melting temperature ($T_d$) was determined at each concentration of GuHCl as the temperature at which $F_{app}$ is equal to 0.5. Plots of $\Delta G^\circ$ as a function of temperature in the transition region led to an estimation of $\Delta H^\circ$ and $\Delta S^\circ$ at $T_d$ for SsAspAT and cpAspAT at each given concentration of GuHCl (Table II).

In Figure 6 the $\Delta H^\circ$ values reported in Table II are presented as a function of $T_d$. The data fit well to a linear dependence, with the slope representing an apparent $\Delta C_p^\circ$. Values of 12 ± 3 and 20 ± 4 kJ.K.mol$^{-1}$ were calculated for the apparent heat capacity change for SsAspAT and cpAspAT, respectively.

**Molecular modeling**

SsAspAT was modeled mainly on the three-dimensional structure template of EcAspAT (PDB code 1ARS) with which it shares the highest sequence similarity (about 19% similarity) compared with other AspATs whose three-dimensional structure has been solved (Mehta et al., 1993).

The sequence alignment between Ss- and EcAspAT (Figure 7) was taken, with minor modifications, from Mehta et al. (1993), who reported a multiple sequence alignment among several aminotranferases from various sources based on care-
Unfolding and molecular modeling analysis of SsAspAT.

Fig. 7. Sequence alignment among pig cytosolic (cpAspAT), E.coli (EcAspAT) and S.solfataricus (SsAspAT) AspATs. Secondary structures defined according to DSSP are reported on lines labeled SS. Symbols are h, b, t and blank for α-helix, β-strand, β-turn, random coil and insertion, respectively. PLP-binding lysine is in bold. Lower-case amino acid symbols denote the segments excluded from the final SsAspAT model. Numbering refers to the cpAspAT sequence.

These two segments belong to the small domain of the 1ARS structure subunit, are exposed to the solvent and relatively distant both from the active site region and the dimer interface, and therefore their removal is expected not to impair substantially the consistency of the model. The overall structure of the molecular model of SsAspAT compares reasonably well with the available AspAT structures (Figure 8).

The quality of the model was assessed with the method of Lüthy et al. (1992). The Ss- and EcAspAT profiles calculated with a 21-residue window were compared. The two profiles are similar up to the region centered in position 219, whereas a negative peak occurs in the region centered around position 232 corresponding to several short insertions/deletions. However, this is the only portion with a negative score. Two more diglycine bridges (Figure 6).
low-scoring regions occur around positions 295 and 343. Stereochemical properties checked with the method of Laskowski et al. (1993) were within the expected ranges.

Inspection of the resulting model allows the following observations at the various levels.

**Molecular surface**
The model predicts a loop exposed to the solvent, particularly accessible in correspondence of Val27. Actually, experiments with limited proteolysis showed that SsAspAT is cleaved at the peptide bond Asn26–Val27 by thermolysin and after residue Lys31 or Lys32 by trypsin (Arnone et al., 1992b). Furthermore, mass spectrometric measurements (Zappacosta et al., 1994) identified the ε-amino groups of Lys 216 and Lys 401 as possible sites of methylation; this is in agreement with the predicted exposed position of the two side chains.

**Active site**
The model of the active site does not show any evident peculiarity. It is interesting, however, that the conserved Ala222, which interacts with the pyridoxal moiety through van der Waals contacts in the mesophilic AspATs, is replaced by the larger Ile residue in SsAspAT. One can speculate that substrate specificity of AspATs is primarily determined by Arg 386 and Arg 292* (* indicates a residue from the other subunit of the dimer molecule), which form ion pairs with the ligand α- and ω-carboxylate groups, respectively. The latter residue is absent in SsAspAT (Birolo et al., 1991) and the model does not indicate any convincing replacement for its function. Similar conclusions have recently been drawn by Okamoto et al. (1996) for the AspAT from *Thermus thermophilus*.

**Dimer interface**
The model of the dimer interface shows interesting features which may explain some aspects of the thermostability of SsAspAT (Figures 9 and 10). A cluster of hydrophobic residues is predicted to occur corresponding to the twofold axis of symmetry. This cluster is formed by the interaction of the side chain of Phe301, which packs against its equivalent from the other subunit, with those of Trp265 and Phe68 from the other subunit (Figure 9). One more hydrophobic cluster can be observed among Phe113 and Ile117 which are in contact with Val294 from the other subunit (Figure 10). A comparison among the dimer interface of the AspAT structures available (in particular cpAspAT, mcAspAT, ccAspAT, EcAspAT) was carried out in an attempt to correlate the evidence obtained in the experiments on thermal and chemical denaturation of the various enzyme forms with the corresponding structural data, including those predicted from the model of SsAspAT presented here. All residues that lose any accessible surface area of subunit association are considered part of the interface (Argos, 1988). Although this criterion is less restrictive than that followed by McPhalen et al., (1992), it was considered sufficiently accurate for comparative purposes. The accessibility calculations were performed in the absence of heteroatoms on apoenzymes. The results of this analysis (Table III) revealed that EcAspAT loses about 16.0% of its total monomer-accessible surface, and mcAspAT 18.0%, ccAspAT 20.0%, cpAspAT 18.0% and SsAspAT 20.0%. Of the lost area, 26.0% in EcAspAT belongs to large and aromatic hydrophobic residues (ILFYWV), 31.0% in ccAspAT, 32.0% in mcAspAT, 33.3% in cpAspAT and 39.3% in SsAspAT. The area lost by charged side chains at the interface (DREKH) follows the opposite trend, being 32.0, 37.4, 36.5, 28.4 and 21.8%, respectively.

**Inner core**
Interestingly, a few charged side chains are predicted to be buried in SsAspAT: Lys79, Arg87 and Glu223. Arg87 can potentially interact with Asp226 and Glu223 to form an H-bond/ion-pair network involving also Asn227, Asn254 and Tyr83. Lys79 can form an H-bond to the hydroxyl group of Tyr269. This suggests also that the core of SsAspAT is less hydrophobic than that of the other mesophilic counterparts.

**Discussion**
In recent years, enzymes purified from thermophilic organisms have attracted much interest, also in consideration of their potential biotechnological applications (Sonntleitner and Fiechter, 1983; Wiegel and Ljungdahl, 1986). Virtually all of the enzymes that have been purified from organisms growing...
Fig. 9. Stereo picture of the SsAspAT (A) dimer interface viewed along the twofold axis of symmetry in the region of the Phe301, compared with the equivalent portions from cpAspAT (B) and EcAspAT (C). Thick and open lines distinguish the two dimer subunits. Residues from the other subunit are labeled B. Numbering refers to Figure 7. Structure is rotated by about 180° along the vertical axis with respect to the view in Figure 10.
Fig. 10. Stereo picture of the \textit{Ss}AspAT (A) dimer interface viewed along the twofold axis of symmetry in the region of the Phe113, compared with the equivalent portions from \textit{cpAspAT} (B) and \textit{EcAspAT} (C). Thick and open lines distinguish the two dimer subunits. Residues from the other subunit are labeled B. Numbering refers to Figure 7. Structure is rotated by about 180° along the vertical axis with respect to the view in Figure 9.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Accessible surface area buried in dimer contacts (%)</th>
<th>Accessible surface area of hydrophobic residues buried in dimer contacts (%)</th>
<th>Accessible surface area of charged residues buried in dimer contacts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{EcAspAT}</td>
<td>16.0</td>
<td>26.0</td>
<td>32.0</td>
</tr>
<tr>
<td>\textit{mcAspAT}</td>
<td>18.0</td>
<td>32.0</td>
<td>36.5</td>
</tr>
<tr>
<td>\textit{ccAspAT}</td>
<td>20.0</td>
<td>31.0</td>
<td>37.4</td>
</tr>
<tr>
<td>\textit{cpAspAT}</td>
<td>18.0</td>
<td>33.3</td>
<td>28.4</td>
</tr>
<tr>
<td>\textit{SsAspAT}</td>
<td>20.0</td>
<td>39.3</td>
<td>21.8</td>
</tr>
</tbody>
</table>

Ile, Leu, Phe, Tyr, Trp, Val were considered to be hydrophobic residues and Asp, Arg, Glu, Lys, His charged residues.
near 100°C are intrinsically more stable than the analogous enzymes purified from mesophilic organisms.

All of the information needed to grant extreme thermal stability to AspAT from *S. solfataricus* is encoded in the gene sequence, since recombinant *SsAspAT* expressed in *E. coli* is functionally equivalent to the enzyme purified from the thermophilic organism (Arnone et al. 1992a); the structural stability of this protein was therefore investigated by analyzing some thermodynamic parameters determined in isothermally and thermally induced denaturation experiments and a molecular model of this enzyme.

Suitable mesophilic counterparts of *SsAspAT* were needed in order to compare thermodynamic and structural data: *EcAspAT* was chosen as the template for molecular modeling because it shares with *SsAspAT* the highest sequence similarity among the AspATs with known three-dimensional structure. No detailed sequence comparison was attempted considering the overall low identity and the fact that even in more favourable cases, no generalities or specific rules of ‘thermophilism’ were found (Böhm and Jaenicke, 1994).

However, *EcAspAT* is not suitable for a thermodynamic comparison with *SsAspAT* since, under the experimental conditions tested, its unfolding pathway proceeds through the formation of two monomeric intermediates (Herold and Kirschner, 1990), whereas the unfolding process of *SsAspAT* can be approximated and treated as a two-state N2 = 2D mechanism.

Under the same conditions, GuHCl-induced unfolding of porcine cytosolic aspartate aminotransferase (cpAspAT) can also be approximated and treated as a two-state N2 = 2D process, and cpAspAT was therefore chosen as the mesophilic counterpart of *SsAspAT* in thermodynamic analysis.

It is intriguing to observe that both *SsAspAT* and cpAspAT, intrinsically more stable than the *E. coli* enzyme to thermal denaturation, show a two-state dimer denaturation in guanidinium chloride whereas *EcAspAT* unfolds along a multistate denaturation pathway with monomeric intermediates.

The comparison of *SsAspAT* model with the *E. coli* enzyme structure revealed a cluster of hydrophobic residues corresponding to the twofold axis of symmetry which may be partially responsible for the increase in thermal stability as well as for stabilizing the dimeric structure against chemical denaturation.

In a recent review on the conformational stability of dimeric proteins, Neet and Timm (1994) suggested that those proteins showing a single transition of native dimer to denatured monomer usually have a greater overall conformational stability than monomeric proteins and that, in many cases, the stabilization energy is provided by the intersubunit interactions. If this is the case, one can suggest a sort of thermophilic evolution starting from *EcAspAT* to *SsAspAT*, with cpAspAT in the middle, involving the stabilization of the intersubunit interactions: the loss of monomer–accessible surface upon dimer formation increases on going from *EcAspAT* to *SsAspAT* through cpAspAT and the relative amount of hydrophobic residues in the lost area goes in the same direction (see Table III). However, these features seem not to be of general and common occurrence.

In fact, a dramatic increase in the amount of buried surface area was found when the inorganic pyrophosphatase from *T. thermophilus* was compared with the corresponding enzyme from *E. coli* (Salminen et al., 1996), but there are many more intermonomer hydrophilic interactions.

A correlation between the increased relative amount of hydrophobic residues buried in subunits interactions and increased thermostability has, instead, been found in a comparative analysis based on glyceraldehyde-3-phosphate dehydrogenase from the hyperthermophilic bacterium *Thermotoga maritima* (Korndörfer et al., 1995). However, these authors found an opposite correlation with the total accessible surface area buried in subunit contacts. An increase in buried hydrophobic surface area in the intersubunits contact region was also found in the triosephosphate isomerase from *Bacillus stearothermophilus* (Delboni et al., 1995). The above examples suggest that different proteins adopt different strategies towards thermostability.

From the thermodynamic analysis, the ΔG° values calculated in the presence of GuHCl were useful both for comparing the stability of the two proteins in the presence of the denaturant and for estimating the free energy difference between unfolded and folded states in the absence of denaturant at 25°C [ΔG°(H2O)]. These calculations showed that *SsAspAT* is more stable than cpAspAT at 25°C, their ΔG°(H2O) values being 70 and 58 J/mol, respectively, whereas the thermophilic enzyme is less stable than its mesophilic counterpart in the presence of denaturant. This finding is in contrast with many reports which correlated thermostability and stability against denaturants (Daniel, 1986; Kristjansson, 1989; Fontana, 1991). Obviously, the two properties are not necessarily associated and must be separately evaluated for different enzymes.

In the binding model proposed by Aune and Tanford (1969) and Makhatadze and Privalov (1992), the difference between the stability of proteins in the absence or presence of GuHCl is proportional to the difference of binding sites, Δn, for GuHCl in denatured and native states, and depends on the activity of the denaturant. In this hypothesis, it can be explained how an enzyme such as *SsAspAT*, even with a higher ΔG°(H2O) (71 kJ/mol) than cpAspAT [ΔG°(H2O) = 55 kJ/mol], can be less stable in the presence of GuHCl because of a higher Δn (29 and 11 for *SsAspAT* and cpAspAT, respectively).

Although the nature of the interaction between proteins and GuHCl has not been completely understood, Makhatadze and Privalov (1992) calculated Δn values for several proteins whose three-dimensional structure was available and tried to correlate these values with the number of residues differentially exposed in unfolded and folded states. This correlation is particularly good with hydrogen bond acceptor residues and more generally with polar residues. Bearing this correlation in mind, it can be suggested that a higher number of polar residues become exposed upon denaturation in *SsAspAT* than in cpAspAT. This in turn could explain the strong influence of GuHCl on the enzyme stability at 25°C and the high Δn value measured for *SsAspAT*.

Moreover, apparent ΔCp, (12 kJ.K/mol) evaluated for *SsAspAT* is lower than that evaluated for cpAspAT (20 kJ.K/mol). It was reported that ΔCp for the unfolding of proteins is roughly proportional to the non-polar surface area which becomes exposed to the solvent upon denaturation (Dill et al., 1989; Privalov and Makhatadze, 1992). Hence the apparent heat capacity change correlates with the number of hydrophobic residues differentially exposed in the native and denatured conformations. This result suggests that *SsAspAT* exposes a minor number of hydrophobic amino acid residues upon denaturation than cpAspAT.

Although triosephosphate isomerase from *B. stearothermophi-
philus has a higher hydrophobicity than other triosephosphate isomerases (Delboni et al., 1995), and the large fragment of Thermus aquaticus DNA polymerase I eliminated unfavourable electrostatic interactions and has a larger hydrophobic core than the Klzenov fragment of DNA polymerase I from E.coli (Korolev et al., 1995), large networks of ion-pairs seem to be the only major determinant of thermostability in indole-3-glycerolphosphate synthase from S.solfataricus (Hennig et al., 1995), and the glutamate dehydrogenase from Pyrococcus furiosus (Yip et al., 1995). Interestingly, in the SsAspAT molecular model some charged side chains are predicted to be buried and to interact to form an H-bond/ion-pair network.

Therefore, SsAspAT seems to adopt a combination of hydrophobic interaction and hydrogen-bonding/salt bridges in order to stabilize its protein architecture, the former being mainly responsible of the stabilization of the quaternary structure and the latter being involved in the cohesion of the protein core and hence in the stabilization of subunit tertiary structure. Moreover, the example of SsAspAT shows how the stability of proteins at high temperatures does not necessarily correlate with the presence of denaturants.

The availability of the structural model of SsAspAT provides a framework for the rational design of site-directed mutagenesis experiments which, in turn, will test the correctness of the predicted structure. Also, the comparison between the expected and the real structure of the SsAspAT, when available, will represent an interesting assessment of the accuracy of the homology modeling techniques applied to cases where low sequence similarity is shared by the model and the template sequences.

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