Truncated aspartate aminotransferase from alkalophilic
*Bacillus circulans* with deletion of N-terminal 32 amino acids
is a non-functional monomer in a partially structured state

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Aspartate aminotransferase (AspAT) from alkalophilic *Bacillus circulans* contains an additional N-terminal sequence of 32 amino acid residues that are absent in all other AspATs from different sources. Modeling suggested that this sequence forms two α-helical segments which establish a continuous network of interactions on the surface of the molecule. In the present study, we studied the role of the N-terminal sequence in folding and stability of AspAT by applying the scanning calorimetry, and CD and fluorescence spectroscopies to the native and truncated enzymes. Truncated AspAT (ΔΔα mutant) devoid of N-terminal residues cannot provide sufficient potential of quaternary intersubunit and subunit-cofactor interactions, which results in a monomeric non-functional conformation. However, the residual tertiary interactions in the ΔΔα mutant are sufficient to: i) provide stability of a residual structure over a wide pH range; ii) confer moderate cooperativity of the denaturant-induced transition while only low cooperativity of the thermal transition, and iii) maintain the hydrophobic core of a part of the structure which prevents aromatic fluorophores from quenching by water. Furthermore, the present study provides evidence that AspAT from the alkalophilic bacterium follows unfolding pathway comprising a stable non-functional intermediate, in contrast to a two-state mechanism of the thermophilic AspAT from *Sulfolobus solfataricus*. Keywords: alkalophilic enzymes/aspartate aminotransferase/differential scanning calorimetry/PLP-dependent enzymes/protein folding

Introduction

Aspartate aminotransferases (AspATs) belong to a wide group of α2-dimeric proteins in the α-family of pyridoxal 5’-phosphate (PLP) -dependent enzymes. Each subunit of AspAT comprises a large domain, a small domain, and an amino-terminal arm (Ford *et al.*, 1980). The N-terminal polypeptide from one subunit protrudes toward the large domain of the other one and is essential for the catalysis (Sandmeier and Christen, 1980; McPhalen *et al.*, 1992). This structure seems to be common for proteins in the α-family (Alexander *et al.*, 1994). We have found that AspAT from an alkalophilic *Bacillus* contains a unique extension of 32 amino acid residues (Battchikova *et al.*, 1996). Fortunately, AspAT provides a perfect experimental system to study the role of this extension since various sequences are available for AspATs from both mesophilic and extremophilic sources and their molecular structures are known in many details.

According to molecular modeling, the N-terminal 32 amino acids of AspAT from *Bacillus circulans* form two α-helices that establish a continuous network of interactions with spatially adjacent structures (Battchikova *et al.*, 1996). This may provide an example of structural ways for adaptation of alkalophilic organisms and their enzyme systems to alkaline conditions. The pH-extremophiles have developed sophisticated mechanisms for survival. An outer cell wall and a membrane play an apparently important role in stabilizing the internal pH. Nevertheless, the pH of a cytosol of alkalophiles can still deviate from neutral by more than two pH units. Previous studies of intracellular enzymes from alkalophiles showed that their structures have unique features and their pH optima are clearly elevated (e.g., see Kobayashi *et al.*, 1982). Therefore, the study of intracellular enzymes is essential for providing further insight into mechanisms of pH-extremophilic adaptation.

In the present study we analyzed by CD and fluorescence spectroscopies and differential scanning calorimetry the conformation of wild-type AspAT in comparison with truncated AspAT devoid of the 32 N-terminal amino acid residues. We showed that the N-terminal sequence of AspAT is crucial for functional quaternary interactions between subunits and/or PLP and significant for the tertiary structure. Deletion of this part of the protein resulted in a non-functional monomer with a partially structured conformational state.

Materials and methods

Protein purification

*Bacillus circulans* var. *alkalophilus* WT AspAT was expressed in *Escherichia coli* and purified as described by Battchikova *et al.* (1996). Mutant AspAT was expressed in *E.coli* as inclusion bodies and purified and refolded by the following procedure. *E.coli* cells were grown in 2 l of LB broth containing ampicillin (100 mg/l) at 37° C until the culture reached the middle log phase. After 3–4 h of growth, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM and the bacterial cells were grown for additional 3 h at 37° C. The cells were harvested by centrifugation at 8000 g for 10 min, suspended in 100 ml of 50 mM Tris–HCl, pH 8.0, containing 0.15 M NaCl and 1 mM phenylmethylsulfonyl fluoride (PMSF), centrifuged as above and washed twice with the same buffer. The cells were resuspended in 50 ml of the above buffer, disintegrated by sonication and centrifuged at 45 000×g for 20 min. The sediment of inclusion bodies typically contained the ΔΔα mutant of AspAT with a purity of 80% (evaluated by SDS–PAGE). The pellet of inclusion bodies containing the ΔΔα mutant of AspAT was solubilized with 6 M guanidine hydrochloride (GdnHCl) in buffer A (50 mM Tris–HCl, pH 8.0) for 2 h at room temperature. After centrifugation for 20 min at 45 000×g, the supernatant was dialyzed...
against 5 M urea in buffer A, then centrifuged to remove a precipitated material and applied on a column (1.7×8 cm) packed with the calcium-tartrate gel prepared according to Akhrem and Drozhdenyuk (1989) and representing a high flow-rate analog of hydroxyapatite. The fraction of adsorbed proteins was eluted with 20–150 mM gradient of sodium phosphate buffer, pH 7.4, containing 5 M urea. The fraction eluted between 50–100 mM was collected and dialyzed against 50 mM sodium phosphate buffer, containing 3 M urea, then against 1 M, 0.5 M, 0.25 M, 0.1 M, and 0.05 M urea and, finally, against 50 mM sodium phosphate buffer, pH 7.4, with an equimolar amount of PLP to obtain the holoprotein or without PLP to obtain the apoenzyme. Every step of dialysis continued for 8–12 h. The purified and refolded protein was centrifuged, concentrated to 8–10 mg/ml and used for further experiments. Typically, the yield of the refolded and purified ΔΔα mutant of AspAT was 20–30 mg from 1 l of the cell culture.

**Enzyme activity assay**

The AspAT activity was measured spectrophotometrically as described previously (Battchikova *et al.*, 1996). The method comprised a coupled assay with malate dehydrogenase in 50 mM MOPS, pH 7.2 at 25 °C with 20 mM L-aspartate and 1 mM 2-oxoglutarate as the substrates. The residual activity as a function of the denaturant concentration was measured as follows: after incubation with GdnHCl for 2 h at 25 °C or 12–16 h at 4 °C, 0.5–2 μl of the protein solution was immediately injected into 1 ml of the substrate mixture in a spectrophotometric cuvette and the slope of the reaction curve was measured.

**Buffer solutions**

The buffer solutions with pH ranging from pH 2–10.7 comprised 0.1 M or 0.05 HEPES, citrate, acetate, phosphate or borate as sodium salts as described in the figure legends.

**Fluorescence measurements**

Intrinsic fluorescence spectra were recorded at 25 °C in 1 cm pathlength cuvette using SFL-1211 fluorometer (Solar, Minsk, Belarus) as previously described in detail (Martsev *et al.*, 1998a,b). Samples for fluorescence measurements were prepared by diluting the protein stock solution to a concentration or pH. The protein fluorescence was excited at 280 or 295 nm with identical results in both cases. The fraction of AspAT unfolded at increasing denaturant concentrations was estimated according to Pantoliano *et al.* (1991) from the maximum wavelength of fluorescence. Before recording emission spectra in the denaturant, protein solutions were allowed to equilibrate for 12–16 h at 4 °C.

**CD spectra**

Circular dichroic (CD) measurements between 200 and 250 nm were carried out at room temperature with a J-20 spectropolarimeter (JASCO, Japan) at AspAT concentration of 0.3–0.4 mg/ml in 50 mM sodium phosphate buffer, pH 7.4, in a cell with 1-mm light path. The results were expressed as the mean residue ellipticity, [θ]_R, which is defined as

\[ [\theta]_R = M \theta_{obs}/(10lc), \]

wherein θ_{obs} is the observed ellipticity in degrees, M is the mean residue molecular mass, l is the length of the light path in centimeters and c the concentration in grams per milliliter (115). A quantitative analysis of CD spectra with predictive calculations of α-helical and other elements in a secondary structure was performed through the neural network approach (Bohm *et al.*, 1992; Andrade *et al.*, 1993; Dalmasso *et al.*, 1994) with the software CD Spectra Deconvolution Program, version 2.1, available on the website: http://bioinformatik.biochtem-te-uni-halle.de

**Calorimetric studies**

Calorimetric measurements were done at heating rate of 60 K/h with DASM-4 differential scanning calorimeter (Biopribor, Pushchino, Russia) equipped with a computer. The protein concentration ranged between 0.3 and 2 mg/ml. In all experiments the reference cell was filled with the buffer used to dialyze the protein sample. The instrumental base-line was subtracted from the heat capacity curves. The base-line was determined prior to each protein scan with both cells filled with the buffer. Three to four scans were recorded for each sample, with variation of enthalpy measurements being within the range of 6–8%. Calorimetric enthalpy was calculated using formalism by Privalov and Khechinashvili (1974) and VSCAL software. Deconvolution analysis of heat capacity curves was performed as described by Privalov and Potekhin (1986) with THERM CALC software. Both VSCAL and THERM CALC software were supplied by the DASM-4 manufacturer.

**Absorption spectra**

The absorption spectra of the enzymes were measured with BioSpec-16105 (Shimadzu, Japan) and Specord M–400 spectrophotometers (Carl Zeiss, Jena, Germany). Concentration of enzymes was determined from the absorption values of 0.1% solution in 1-cm pathlength cuvette at 280 nm. The absorption values 0.988 and 0.876 for WT AspAT and the ΔΔα mutant protein, respectively, were calculated from the known amino acid sequences according to Gill and von Hippel (1989).

**Other methods**

SDS–PAGE was performed according to Laemmli (1970) and stained with Coomassie Brilliant Blue R-250. Gel filtration analysis of WT and the ΔΔα mutant was carried out on a TSK-gel G 3000 SWXL column (7.8×300 mm) (TosoHaas, USA). The column was calibrated with the following proteins of known molecular weight: α-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome c (12.4 kDa). Values of 50 μl of AspAT (0.05–0.1 mg) were loaded on the column and isocratically eluted with 50 mM sodium phosphate buffer, pH 7.4, containing 0.1 M NaCl. All experiments were carried out at 25 °C at a flow rate of 60 ml/h.

**Results**

**General properties of WT AspAT and ΔΔα mutant**

WT AspAT from *B. circulans* expressed in *E. coli* host cells was purified from cytosolic extracts to apparent homogeneity as judged by SDS–PAGE. The specific activity of the WT AspAT was 80 units/mg protein, consistent with that previously described for this enzyme (Battchikova *et al.*, 1996). Comparison of a molecular mass of WT AspAT estimated from gel chromatography (96±2 kDa, Figure 1) and SDS–PAGE (47±3 kDa) indicates that WT AspAT is a homodimer.

The ΔΔα mutant of AspAT was expressed in *E. coli* as an insoluble polypeptide packed into inclusion bodies under physiological conditions of bacterial growth. This finding suggests higher aggregation propensity of the mutant protein, which may be considered as indirect evidence of its non-native conformation and different conformational states of the WT
The far-UV CD spectra of WT AspAT and the Δ2α mutant showed minima of the mean residue ellipticity at 208 nm and 215 nm (Figure 3), which is typical for proteins with the high content of an α-helical structure (Chen et al., 1978). However, the Δ2α mutant exhibited a lower negative ellipticity, which is attributable to a lower amount of α-helices in comparison with WT AspAT. The analysis of the spectra with the CD Spectra Deconvolution Program yielded 33.8% α-helix and 29.9% random coil for WT AspAT versus 23.4% of α-helix and 39.4% random coil calculated for the Δ2α mutant. These results are consistent with the model of WT AspAT which proposed the existence of two α-helices in the additional N-terminal sequence formed by the 32 amino acid residues (Battchikova et al., 1996). In the presence of 4 M GdnHCl, virtually no secondary structure was observed in the CD spectra of the two proteins.

### Absorption spectra and bound cofactor

Deletion of the N-terminal sequence and removal of PLP should be noted that a gradual increase in the emission maxima of apo- and holo-forms did not display a shift in wavelength on varying the pH. Addition of GdnHCl resulted in a significant decrease in the fluorescence intensity which indicates the accessibility of tryptophanyl fluorophores to the solvent. Noteworthy, the spectra of unfolded WT AspAT or the Δ2α mutant were virtually identical, thus indicating that the two denaturant-unfolded proteins have the same extent of solvent exposure of Trp residues and that the difference in emission intensity observed without a denaturant resulted from the tertiary structure rather than from the N-terminal deletion.

### pH stability of the WT and mutant forms of AspAT

pH stability of WT AspAT and the Δ2α mutant was analyzed in the pH range of 2–10.7 by measuring the intrinsic fluorescence of the proteins (Figure 5). At pH values between 3.5 and 5.0, close to the calculated isoelectric point (pI ~4.5), the two proteins produced a small amount of aggregates with a slight turbidity of the solution. Therefore, we did not analyze conformational changes within this pH range. Variation in the pH range 2–10.7 did not produce any significant effect either on the fluorescence intensity or the emission maximum. It should be noted that a gradual increase in the emission maximum of the WT enzyme (from 335 nm to 342 nm) occurred between pH 2 and 10.7. This finding suggests that tryptophanyl residues of WT AspAT are more exposed to the solvent in alkaline than in acidic pH. In the mutant protein, the emission maxima of apo- and holo-forms did not display a significant shift in wavelength on varying the pH. Addition of GdnHCl resulted in a significant red shift of the emission maximum even at pH 10.7, which indicates that in the pH range from 2–10.7 both WT AspAT and the Δ2α mutant retain a compact conformation capable of the transition to a more unfolded state (see Figure 5). Together, these data suggest high pH-stability of microenvironment of tryptophanyl fluorophores for both the WT and mutant protein.

### GdnHCl-induced unfolding of WT AspAT and the Δ2α mutant

Stability against GdnHCl-induced unfolding was estimated by recording fluorescence spectra of WT AspAT and the Δ2α mutant.
mutant in the presence of increasing concentrations of GdnHCl. Denaturation of both WT AspAT and the Δ2α mutant in 6.0 M GdnHCl resulted in a red shift of the emission peak (excitation at 280 nm) from 338–339 nm to 352–354 nm with a significant (~3-fold) decrease in the fluorescence intensity (see Figure 4). The emission spectra of the two proteins incubated in 4, 5 or 6 M GdnHCl were superimposable. The denaturation curves were remarkably similar for the proteins (Figure 6), with virtually the same $D_{30}$ values (1.9–2.0 M), the denaturant concentration providing half-maximal unfolding. Denaturant-induced unfolding curves demonstrated that conformational stability of a residual structure in the truncated AspAT mutant is virtually identical to the stability of the WT enzyme, even though the mutant protein was monomeric and did not show the functional activity.

**GdnHCl-induced denaturation of WT AspAT as monitored by activity**

Disruption of functionally significant tertiary/quaternary interactions in WT AspAT was studied by incubating the enzyme at different concentrations of GdnHCl and measuring the residual transaminase activity. GdnHCl-induced denaturation of WT AspAT demonstrated a significant extent of reversibility because removal of the denaturant by dilution or dialysis led to a recovery of up to 80% of the initial activity (Figure 7, inset). Hysteresis that is observed between the denaturation/
AspAT with N-terminal deletion

Fig. 5. pH-stability of WT AspAT and its Δ2α mutant monitored by fluorescence spectroscopy with excitation at 280 nm. The emission spectra were recorded in 0.1 M sodium citrate (pH 2–4.5), sodium acetate (pH 5–5.5), sodium phosphate (pH 6–8), and sodium borate (pH 8.5–10.7). A stock solution of protein (9–10 mg/ml) was diluted in the corresponding buffer to a final concentration of 50 μg/ml followed by incubation for 16–18 h at 4 °C. Solid triangles indicate the emission maximum at 352 nm for WT AspAT unfolded in 6 M GdnHCl, pH 7.

Fig. 6. GdnHCl-induced unfolding curves of WT AspAT and the truncated Δ2α mutant. Fluorescence wavelengths were obtained upon excitation at 280 nm. Denaturation was carried out for 16–18 h at 4 °C in 50 mM MOPS, pH 7.2, at the indicated concentrations of GdnHCl.

Fig. 7. GdnHCl-induced inactivation and unfolding of WT AspAT monitored by the enzyme activity assay and fluorescence spectra. The fraction of the folded protein was estimated from the fluorescence maximum wavelength according to Pantoliano et al. (1991). The fluorescence spectra were recorded with excitation at 280 nm after incubation of protein samples for 12–16 h with the indicated concentrations of GdnHCl at 4 °C in 50 mM MOPS, pH 7.2. Inset: inactivation and reactivation curves for WT AspAT obtained by measuring the residual activity. In the inactivation assay, an aliquot (0.5–2 μl) of the protein, incubated with varying GdnHCl concentrations, was mixed with 1.0 ml of the substrate solution and the enzyme activity was measured. In the reactivation assay, the samples of the protein, dissolved in 6 M GdnHCl, were diluted to the required denaturant concentration, then incubated with lower GdnHCl concentrations, followed by injection of an aliquot into the substrate solution.

refolding resulted in a renaturation observed at the lower denaturant concentrations.

The loss of activity of WT AspAT was observed at the lower concentrations of GdnHCl ([D]050 = 0.9 M) than the unfolding concentrations measured by fluorescence, [D]050 = 1.9 M, (see Figure 7). This loss of activity can be related to disruption of intersubunit interactions and/or dissociation of the cofactor, rather than to global unfolding. As another possible explanation, one can assume local unfolding of a spatially restricted but functionally essential structure in the active site. At the intermediate denaturant concentrations that lie between the concentration of essentially complete inactivation and the concentration of fluorescence-revealed unfolding, a non-functional unfolding intermediate of AspAT is highly populated. For example, at 1.5 M GdnHCl, the population of inactive AspAT is about 85% (see Figure 7). At this GdnHCl concentration, a rough estimate for the fraction of the unfolded enzyme gives approximately 25%. Thus, the roughly estimated population of the intermediate is about 60% at 1.5 M GdnHCl. The lack of significant fluorescence changes at this concentration of the denaturant indicates that conformational changes involved in the formation of the intermediate do not alter an environment of the aromatic fluorophores.

The GdnHCl-induced denaturation studies were previously performed for several aspartate aminotransferases, which permits a comparison of some features of their folding and stability. Arnone et al. (1997) reported that AspAT from
S. solfataricus and from pig heart in the presence of GdnHCl denatured through a two-state process. However, unfolding pathway of AspAT from E. coli proceeded through the formation of two non-functional monomeric intermediates (Herold and Kirschner, 1990). Interestingly, stabilities of the two groups of structure-forming interactions, namely, the intersubunit and subunit-cofactor interactions, on one hand, and intrasubunit interactions, on the other hand, are similar for our WT AspAT and AspAT from E. coli that follows the unfolding pathway involving intermediates. Furthermore, WT AspAT from B. circulans appeared to be considerably more stable than mitochondrial AspAT from pig heart that lost 50% of its activity at GdnHCl concentrations as low as 0.5 M and underwent spectroscopically revealed unfolding between 1 and 2 M GdnHCl (West and Price, 1989).

**Thermodynamic stability of WT and mutant AspATs**

The differential scanning calorimetry of WT AspAT at pH 7.4 (Figure 8) demonstrates a single peak of thermal unfolding with $T_m$ around 79 °C. A degree of reversibility determined in repetitive cycles of heating and cooling protein samples in the calorimetric cell was very low after complete thermal unfolding but gradually increased as the temperature of the first scan decreased below the $T_m$. Despite this apparent irreversibility, a thermodynamic analysis can be applied to this process as outlined by Manly et al. (1985), Hu and Sturtevant (1987), and Brandts et al. (1989) if the phenomena causing irreversibility (generally, protein aggregation at near-transition temperatures) are much slower than the unfolding transition. This criterion was met with our experimental system. Measurement of the specific calorimetric enthalpy, $\Delta H$, gave a value of 4.4 cal/g (426 kcal/mol) which falls within the range generally observed for completely compact proteins. The ratio of the calorimetric to van’t Hoff enthalpies (the cooperativity ratio, $CR = H_{cal}/H_{vH}$, with both enthalpies calculated per mole of the homodimer of 94 kDa) was about 3, which is indicative of the presence of intermediates of thermal unfolding at neutral pH.

Unlike WT AspAT, the $\Delta 2\alpha$ mutant showed the low cooperative unfolding transition between 40 and 70 °C, with $\Delta H$ of 0.66 cal/g for the holoprotein (see Figure 8) and 0.92 cal/g for the apoprotein (not shown). Noteworthy, the specific heat capacity, $C_p$, of the mutant enzyme at 25 °C was 0.40 ± 0.05 cal/(K g) as measured for several protein samples. This $C_p$ value is intermediate between $C_p$ (25 °C) of native WT AspAT [0.29 ± 0.05 cal/(K g)] and $C_p$ of the unfolded proteins determined at 25 °C from the second scan [0.53 ± 0.06 cal/(K g)] for mutant AspAT and 0.48 ± 0.07 cal/(K g) for WT AspAT. The intermediate $C_p$ values of the compact mutant enzyme at near-physiological conditions, together with its low unfolding enthalpies, strongly suggest that the $\Delta 2\alpha$ mutant of AspAT generates, under physiological conditions, a partially structured state with significant loss of tertiary interactions. This partial loss of the tertiary structure is consistent with a decrease in the amount of a secondary structure demonstrated by the CD spectroscopy (see Figure 3).

**Partially structured conformation of truncated AspAT is not artefact of refolding conditions**

Our data comprise sufficient evidence that a partially structured state of the $\Delta 2\alpha$ mutant cannot result from an inadequate refolding procedure after unfolding of the protein in the denaturant during preparation from the inclusion bodies. Firstly, we used several protocols for refolding of the mutant enzyme with variable pH (pH 7–9), additions of dithiothreitol, and the presence or absence of cofactor, PLP. These different procedures resulted in proteins of virtually identical spectroscopic and calorimetric properties of the refolded truncated protein. Secondly, unfolding of the truncated protein was a completely reversible process, as judged by the CD and fluorescence spectroscopy after the second round of denaturant-induced unfolding-refolding (data not shown). This strongly suggests that incomplete folding of truncated AspAT is an intrinsic feature tightly associated with the N-terminal deletion rather than a consequence of an inadequate refolding procedure.

**Discussion**

WT AspAT from alkalophilic B. circulans is a protein that possesses a significant amount of $\alpha$- helices and a well-defined tertiary structure, which form native quaternary interactions including the specific subunit and cofactor association. In this bacterial AspAT, the N-terminal 32 amino acids constitute an $\alpha$-helix/PLP interaction, on the other hand, and intrasubunit interactions, on the other hand, are similar for our WT AspAT and AspAT from E. coli that follows the unfolding pathway involving intermediates. Furthermore, WT AspAT from B. circulans appeared to be considerably more stable than mitochondrial AspAT from pig heart that lost 50% of its activity at GdnHCl concentrations as low as 0.5 M and underwent spectroscopically revealed unfolding between 1 and 2 M GdnHCl (West and Price, 1989).

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Detailed analysis of calorimetric data provides further arguments in favor of the partially structured state of truncated AspAT. Although the partial heat capacity, $C_p$, of this state in a pre-transition region [0.40 ± 0.05 cal/(K g)] at 25 °C is
consistent with the Cp values previously observed for many unfolded proteins (Privalov, 1979; 1982), this Cp (25°C) value for the mutant protein is still lower than Cp of the thermally-unfolded states obtained for the WT enzyme [0.48 ± 0.07 cal/(K g)] or Δ2α mutant [0.53 ± 0.08 cal/(K g)]. This indicates, firstly, that the Δ2α mutant exposes, at near-physiological temperatures, a larger amount of hydrophobic residues than the WT enzyme, as could be expected for a partially unfolded protein. Moreover, the exposure of hydrophobic residues in the Δ2α mutant below the transition temperature is still incomplete and some of these residues are involved into a hydrophobic core. Retention of a hydrophobic core involving tryptophanyl residues is indicated by the emission maximum at 338 nm in the fluorescence spectrum of the truncated protein. Despite the clear-cut differences between WT AspAT and the Δ2α mutant in their tertiary structures, these structures remained essentially unchanged within the pH range of 2–10.7 (Figure 5) and displayed virtually identical stability against chemical denaturant (Figure 6). However, fluorescence results, by virtue of their inherent limitations, cannot be directly related to the amount of structure that undergoes an unfolding transition. It is conceivable that the smaller amount of structure in truncated AspAT displays stability similar to that of WT AspAT. Furthermore, poor discrimination of structurally distinct proteins in stability when employing GdnHCl is frequently observed. This is consistent with the results by Arnone et al. (1997) for AspAT from *S.solfataricus*, as well as with the general concept that mechanisms of thermal and denaturant-induced unfolding differ significantly and may involve different structural units of a protein (Makhatadze and Privalov, 1992). Therefore, the stability parameters obtained in denaturant- and thermally-induced experiments should not be generally considered as interrelated quantities; rather, they seem to represent independent thermodynamic estimates of the two different unfolding processes.

A partially structured and non-functional conformation of truncated AspAT provides the experimental evidence for the computer model predicting the existence of the N-terminal 32 amino acid residues in the form of two α-helices forming a continuous network of stabilizing interactions on the surface of AspAT from *B.circulans* (Battchikova et al., 1996). We have demonstrated that the N-terminal sequence is critical for quaternary interactions that provide function and constitutes a significant, if not crucial, determinant of tertiary structural interactions. Furthermore, the present work provides proof that AspAT from the alkalophilic bacterium follows an unfolding pathway comprising a stable intermediate, in contrast to the two-state mechanism found for thermophilic AspAT from *S.solfataricus* (Arnone et al., 1997). Our results are thus in agreement with the pathway comprising related intermediates as demonstrated by Herold and Kirschner (1990) for AspAT from *E.coli*.

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### References


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