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Structural Stability and Functional Analysis of L-Asparaginase from *Pyrococcus furiosus*

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Abstract—We report studies on an L-asparaginase from *Pyrococcus furiosus*, cloned and expressed in *Escherichia coli* and purified to homogeneity. Protein stability and enzyme kinetic parameters were determined. The enzyme was found to be thermostable, natively dimeric, and glutaminase-free, with optimum activity at pH 9.0. It showed a K_m of 12 mM and a substrate inhibition profile above 20 mM L-asparagine. Urea could not induce unfolding and enzyme inactivation; however, with guanidine hydrochloride (GdnCl) a two-state unfolding pattern was observed. Reduced activity and an altered near-UV-CD signal for protein at low GdnCl concentration (1 M) suggested tertiary structural changes at the enzyme active site. A homology three-dimensional model was developed and the structural information was combined with activity and stability data to give functional clues about the asparaginase.

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Key words: *Pyrococcus furiosus*, L-asparaginase, hyperthermophile, chemical denaturation, homology model, stability, enzyme kinetics

L-Asparaginase (L-asparagine-amidohydrolase, EC 3.5.1.1) catalyzes the conversion of L-asparagine into aspartic acid and ammonia. As a chemotherapeutic agent, the enzyme is used in the treatment of acute lymphoblastic leukemia. It functions by reducing the availability of circulatory L-asparagine to tumor cells [1]. L-Asparaginase has been used for making a diagnostic biosensor as the amount of ammonia produced by the action of the enzyme directly correlates to the level of L-asparagine in a patient's blood [2]. Asparaginases obtained from bacteria have been categorized as type I and type II enzymes based on their subcellular location and their kinetic properties. Type I asparaginases are constitutively expressed cytoplasmic enzymes with characteristic specificity for L-asparagine, while type II are regulatory and periplasmic enzymes with a wider substrate specificity [3, 4]. To date, asparaginases from *Escherichia coli* (EcAII) and *Erwinia chrysanthemi* (ErA) have been used for clinical purposes. However, these enzymes have an associated glutaminase activity, which causes side effects [5, 6]. Further, these enzymes have low stability and a reduced half-life in the blood, requiring multiple dose administration for effective treatment [7-10].

Recent advances in food technology have demonstrated that fried and baked food (particularly fried potato) contains a significant amount of acrylamide (a carcinogenic toxicant) [11, 12], formed by the reaction of asparagine with reducing sugars [13]. A pretreatment of potato slices and bread dough with asparaginase before frying or baking prevents acrylamide formation [14-16]. In this regard, asparaginases from *Aspergillus oryzae* and *A. niger* are in use in baking industries [14, 17]. These enzymes work optimally at 40-60°C and pH 6.0-7.0. Since baking temperatures often go up to 120°C, it is desirable to have enzymes that are stable and active over a wide range of temperature and pH. Therefore, L-asparaginases from various sources (bacterial, fungal, plant, and animal) have been investigated [18-22].

For both therapeutic and industrial applications, stability emerges as a key factor determining applicability of L-asparaginase. Attempts made to confer stability include

Abbreviations: AMC, 7-amino-4-methylcoumarin; EcAII, ErA, PfA, PhA, TaqA, and TthA, asparaginases from *Escherichia coli*, *Erwinia chrysanthemi*, *Pyrococcus furiosus*, *P. horikoshii*, *Thermus aquaticus,* and *T. thermophilus*; GdnCl, guanidine hydrochloride.

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entrapment of the enzyme in liposomes or microcapsules [23, 24], covalent coupling with dextran, albumin, or monomethoxy-PEG [25-27], and mutational approaches [8, 28, 29]. In this regard, archaeal asparaginases from *Thermus aquaticus* (TaqA), *T. thermophilus* (TthA), *Tetrahymena pyriformis*, and *Bacillus stearothermophilus* appeared promising because of their inherent thermostability and substrate specificity [30-32].

The present study is a report about cloning, expression in *E. coli*, and purification of an L-asparaginase of *Pyrococcus furiosus* (PfA) (1,888,256-1,889,236 bp *ansA* (*H*) genes). The purified enzyme was characterized for its optimum substrate concentration, pH, and temperature of activity. The enzyme displayed no glutaminase activity. *In vitro* unfolding/refolding was studied in the presence of guanidine hydrochloride (GdnCl) and urea. The protein displayed a two-state unfolding transition in the presence of GdnCl and refolded back, regaining 100% activity. Taking coordinates from a recently published structure of a homologous L-asparaginase of *Pyrococcus horikoshii* (PhA) [33], a model of PfA has been developed. Activity and stability data when compared to the structural model indicated the presence of Tyr residues at the enzyme active site. Based on our observations a mutational approach is suggested to obtain a thermostable, mesoactive protein.

MATERIALS AND METHODS

Cloning and expression of PfA. A putative L-asparaginase gene consisting of a 981-bp open reading frame was amplified by PCR from the *P. furiosus* genomic DNA (a gift from Dr. P. Guptasarma) using primer pair 5′-GGC GGG ATC CTA ATC TCT AAG CTC TCC-3′ and 5′-GTG CAG CAT ATG AAA ATT CTT CTA ATT G-3′. Following double digestion by *Nde*I and *Bam*HI, the PCR product was ligated in a pET14b expression vector and introduced into *E. coli* Codon Plus Rosetta strain (Stratagene, USA). Actively growing recombinant cells (A_{600} of 0.6-0.8) were induced with 1 mM isopropyl-β-D-thiogalactopyranoside and harvested 6 h post induction. Expression was analyzed on SDS-PAGE.

Purification of PfA. Affinity purification under denaturing condition was done after sonication of harvested cell pellet in buffer A (10 mM Tris-HCl, 100 mM NaH_2PO_4, 6 M GdnCl), pH 8.0. Following centrifugation the supernatant was passed through a 3 ml Ni-NTA agarose column (Qiagen, USA) and washed with three column volumes of buffer A, pH 6.3. Protein was eluted first at pH 5.9 and then at pH 4.5 using buffer A. Fractions were analyzed by SDS-PAGE and dialyzed against buffer B (50 mM Tris-HCl, 100 mM NaCl, pH 8.0). The dialyzed sample was heated at 85°C for 15 min and centrifuged to remove thermally aggregated proteins. The supernatant containing soluble protein was further passed through a DEAE column (GE-Healthcare, USA) and eluted with a gradient of NaCl on an AKTA Explorer chromatography system. Eluted protein was dialyzed against buffer B, concentrated, and run on SDS-PAGE. Protein concentration was determined by Bradford's method [34] and by using ε_{280} = 27,390 $M^{-1} \cdot cm^{-1}$.

Determination of molecular mass of PfA. Molecular mass of the purified protein was determined by denaturing gel electrophoresis (12% SDS-PAGE) and MALDI-TOF mass spectrometry. For the native enzyme, molecular mass was determined by analytical gel filtration chromatography. Protein solution (8.5 μg) was loaded on a Superdex-200 analytical gel filtration column (32/30; GE Healthcare) on a SMART chromatographic workstation. The column was equilibrated and protein was eluted with buffer B.

Assay of L-asparaginase. L-Asparaginase activity was measured by a standard method using Nessler's reagent [35]. In short, reaction mixture containing 50 mM Tris-HCl, pH 9.0, 10 mM L-asparagine (Merck, Germany) and varying amount of enzyme solution in a final volume of 2 ml was incubated for 10 min at 80°C. After incubation, the reaction was stopped by adding 100 μl of 1.5 M trichloroacetic acid. The solution was centrifuged followed by addition of 1 ml Nessler's reagent to 500 μl of the supernatant diluted with 7 ml of water. The absorbance A_{480} of the resulting solution gave a measure of enzyme activity. A standard curve was prepared with ammonium sulfate. To determine the effect of temperature and pH, enzyme activity was measured at varying temperatures (37-95°C) and varying pH (4.0-10.5), other factors remaining unchanged. One international unit (IU) of L-asparaginase activity is defined as the amount of enzyme liberating 1 μmol NH_3 in 1 min at 80°C under the above-mentioned conditions. Specific activity of L-asparaginase is defined as the units per milligram protein.

A fluorescence-based assay method was used for determining activity of GdnCl-containing samples [36, 37]. In the assay, 10 μl of 10 mM L-aspartic acid β-(7-amido-4-methylcoumarin) (Asp-AMC) in ethylene glycol, 90 μl of 50 mM Tris-HCl (pH 9.0), and 100 μl of enzyme were incubated at 80°C for 10 min. The fluorescence of free 7-amino-4-methylcoumarin (AMC) liberated by the reaction was measured using a Perkin Elmer LS 55 B spectrofluorimeter (USA) with excitation and emission monochromators fixed at 350 and 450 nm, respectively. A standard curve was plotted with AMC. One unit of asparaginase is defined as the amount of enzyme that liberates 1 μmol of AMC from the substrate per minute at 80°C.

Kinetic analysis. Steady-state enzyme kinetics was measured in triplicates in 50 mM Tris-HCl, pH 9.0, by varying the concentration of the substrate (0.5-80 mM L-asparagine). The kinetic parameters K_m, k_{cat}/K_m (kinetic efficiency), and K_s (substrate inhibition constant) were

calculated and data fitted by nonlinear regression analysis of steady-state rate for the substrate inhibition model.

Chemical denaturation. For equilibrium unfolding studies, protein samples (4.5 μM) were made with varying concentrations of urea (0-8 M) and GdnCl (0-6 M) and incubated overnight. Fluorescence of incubated samples (taken in 1 cm quartz cuvette) was recorded as an emission scan from 300 to 400 nm with excitation monochromator set at 280 nm. Fluorescence intensities at 339 nm were recorded for each sample. Data were plotted as % unfolding against increasing denaturant concentrations taking intensity for the native protein (no denaturant) as 0% and for the fully denatured protein as 100%. A similar plot was obtained for far-UV-CD data where samples were taken in a 2 mm quartz cuvette and scanned over 250-200 nm using a Jasco-810 spectropolarimeter (Japan). Signal at 222 nm, averaged over three scans, was taken as the datum point. For monitoring tertiary structural changes, near-UV-CD data were collected over 350-250 nm in the spectropolarimeter for GdnCl-incubated protein samples (1.6 mg/ml) taken in a 1 cm quartz cuvette.

RESULTS

Protein expression and purification. Recombinant L-asparaginase of *P. furiosus* was induced and overexpressed in *E. coli* host. The protein was purified to homogeneity in three consecutive steps of affinity chromatography, thermal denaturation, and ion-exchange chromatography. The pure enzyme displayed a single 37 kDa band on a 12% SDS-PAGE and a 37.86 kDa peak by mass spectrometry (Fig. 1a). The protein eluted as a single 75.45 kDa peak from the Superdex-200 column, indicative of being natively dimeric (Fig. 1b).

Enzyme activity as a function of pH and temperature. An increase in enzyme activity was observed with increase in pH up to a maximum of 550 IU/mg at pH 9.0 followed by a sharp fall (Fig. 2a). A clear temperature optimum could not be assigned to the enzyme as its activity progressively increased with increase in temperature up to and beyond 85°C (Fig. 2b). The logarithm of specific activity at each temperature was plotted against the reciprocal of absolute temperature (Arrhenius plot) to determine the activation energy (E_a) using Eq. (1):

$$\ln V = \ln A - E_a/RT\,, \qquad (1)$$

where R is the gas constant and A is the pre-exponential factor. From the plot, the activation energy was found to be 42.5 kJ/(mol·K) (Fig. 2b, inset). The enzyme incubated for different time periods monitored over a range of 55-85°C showed linear kinetics (Fig. 2c).

Enzyme kinetics as a function of substrate concentration. Enzyme activity was monitored as a function of substrate concentration [S] and plotted (Fig. 2d). The data were fitted by nonlinear regression analysis of steady-state rate for the substrate inhibition model (Eq. (2)) to determine the kinetic parameters K_m and K_s:

$$v = \frac{V_{max}\,S}{K_m + S\,(1 + S/K_s)}\,. \qquad (2)$$

The kinetic parameters for PfA with L-asparagine as a substrate were as follows: $k_{cat} = 870 \pm 170$ sec^{-1}, $K_m = 12 \pm 4$ mM, $k_{cat}/K_m = 72{,}500$ M^{-1}·sec^{-1}, $K_s = 125 \pm 77$ mM. Unlike typical Michaelis–Menten behavior, the activity of the enzyme was inhibited above 20 mM L-asparagine.

Determination of stability from chemical denaturation studies. Protein samples incubated with urea did not exhibit unfolding. This was indicated by unchanged CD and fluorescence signals when plotted against increasing concentrations of urea (Fig. 3a). In the presence of GdnCl, however, the curves followed a sigmoidal pattern

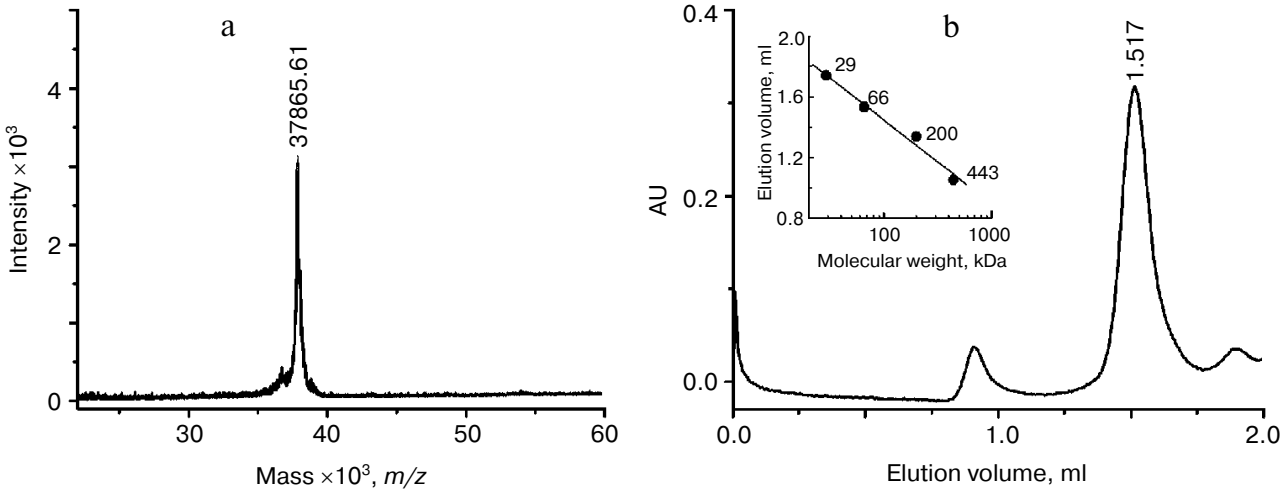

Fig. 1. a) Mass spectrum of purified PfA. b) Gel filtration chromatogram with standard graph for the column (inset).

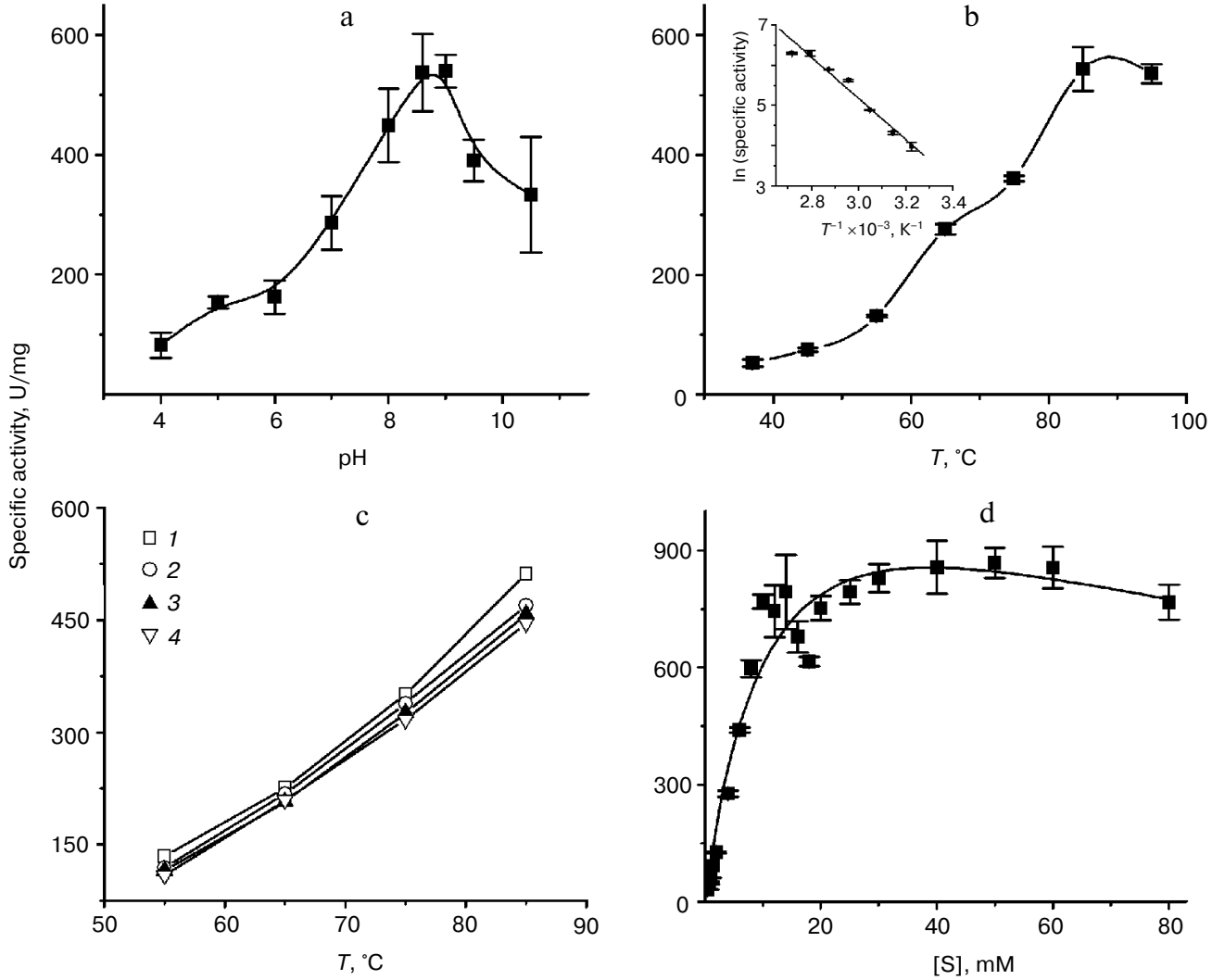

Fig. 2. Determination of enzyme activity parameters. Specific activity at different pH values (a) and different temperatures (b). For pH dependence, the buffers used (0.05 M) were: sodium acetate (pH 4-5), sodium phosphate (pH 6-7), Tris-Cl (pH 8.0-9.5), and Na_2HCO_3/NaOH (pH 10.5). The inset is an Arrhenius plot for activation energy. c) Temperature profile of PfA incubated for 2 (*1*), 5 (*2*), 7 (*3*), and 10 min (*4*). d) Dependence of enzyme activity on substrate concentration. The curve is a nonlinear fit using the equation given in the text to get K_m values.

with unfolding beginning at 3.25 M GdnCl and complete unfolding achieved at 4.75 M GdnCl. As expected, the enzymatic activity of urea-containing protein samples remained unchanged. In contrast, the GdnCl-incubated samples showed a significantly lower activity even with 1 M GdnCl, followed by a sharp decrease in activity with increasing denaturant concentrations (Fig. 3b). The GdnCl unfolding data fitted well to the two-state unfolding model given by Eq. (3):

$$K = (y_N - y)/(y - y_D), \quad (3)$$

where K is the equilibrium constant, y is the observable used to monitor unfolding, and y_N and y_D are its values for the native and the denatured states, respectively. The ΔG_{H_2O} value was obtained by plotting the linear portion of the fit to the equation and extending to zero concentration of GdnCl (Fig. 3c). The other stability parameters obtained from this fit are as follows: $\Delta G_{H_2O} = 12.0$ kcal/mol, $[GdnCl]_{1/2} = 3.86$ M, slope of the plot -3.112, $r = -0.99872$, $P < 0.0001$.

Determination of tertiary structural changes with denaturant. To find a reason for decreased activity at low GdnCl concentrations, a near-UV-CD spectrum was obtained for protein sample containing 1 M GdnCl. A clearly shifted peak as compared to the native protein was observed around 280-290 nm indicating tertiary structural changes involving tyrosine residues (Fig. 3d).

DISCUSSION

Proteins obtained from thermophiles are generally stable owing to their evolutionary adaptation to extremes

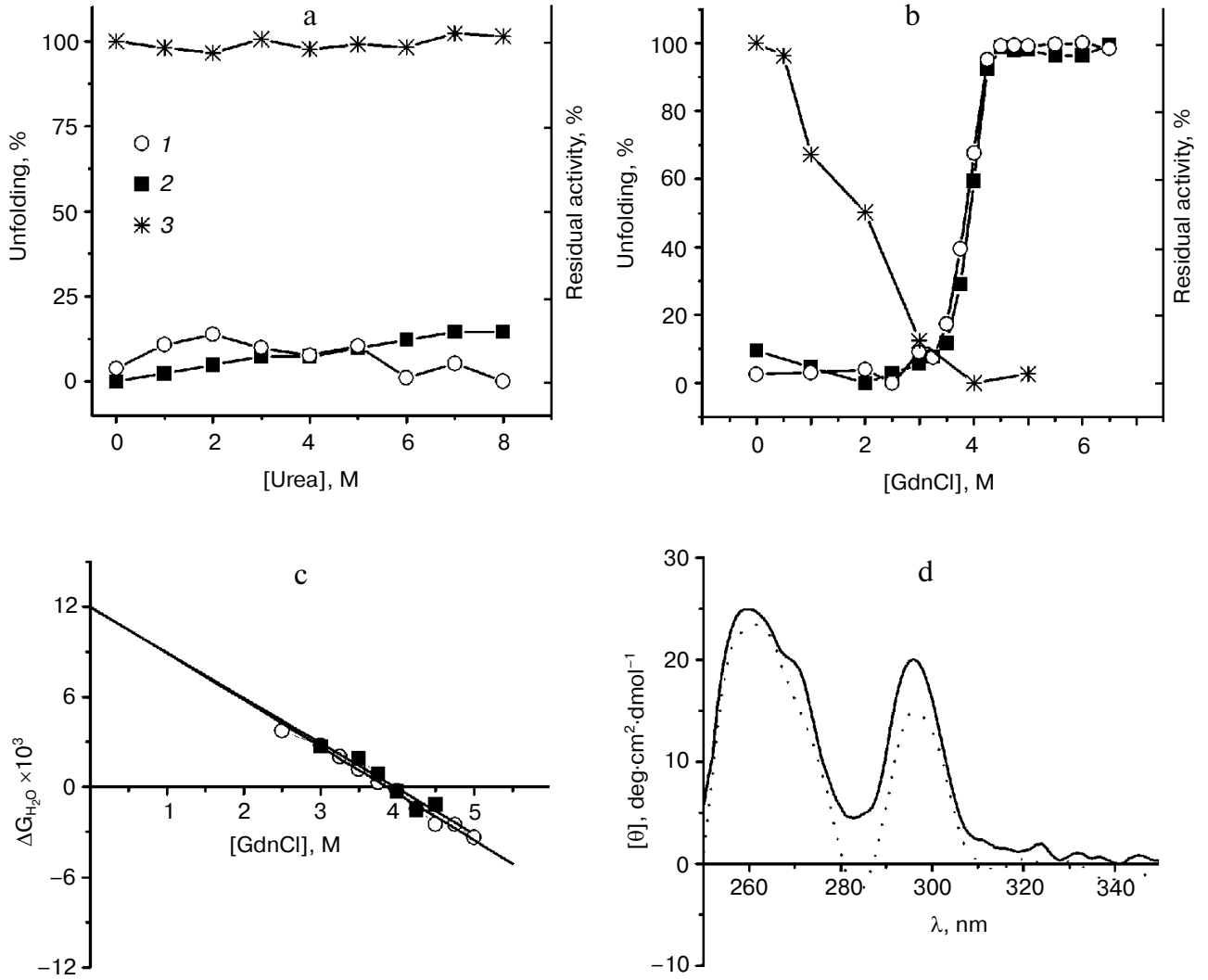

Fig. 3. Determination of stability parameters of PfA. Chemical denaturation monitored by CD (*1*) and fluorescence (*2*) for protein incubated with urea (a) and GdnCl (b), respectively; *3*) residual activity (%) at each concentration. c) ΔG_{H_2O} calculated from the linear part of GdnCl unfolding curve as monitored by CD and fluorescence. d) Near-UV-CD spectrum of non-incubated (solid curve) and 1 M GdnCl-incubated PfA samples (dotted curve).

of temperature. Therefore, apart from being subject of protein folding studies, thermophilic proteins are commercially used as stable enzymes in a variety of industrial applications [38, 39]. As stated earlier, L-asparaginases from mesophilic sources are somewhat unstable to meet expanding uses of this important enzyme [9, 10], motivating a search for stable alternative sources. With the idea that L-asparaginase from a thermophilic source will be inherently stable, we cloned PfA in *E. coli* and expressed and characterized the enzyme.

The protein folded without aggregation following affinity purification under denaturing conditions. With a molecular weight of 75.45 kDa, the native protein appeared as a dimer. Asparaginases from other sources are reported to occur in various forms, viz., dimeric [33], tetrameric [18, 20, 21, 40], and hexameric [31]. Depending on the multimericity, enzymes obtained from different sources are reported to have varied enzymatic parameters. For example, EcAII and ErA are natively tetrameric having activities between 200-650 IU/mg and K_m ranging between 10-12 μM [21, 41]. On the other hand, specific activities reported on thermophilic L-asparaginase are of the order of 600-850 IU/mg with K_m values ranging within 2.8-8.6 mM [30-32]. Corresponding values for PfA were found to be higher than expected (K_m = 12 mM and specific activity of 500 IU/mg) (Fig. 2d). Such values indicate that the structural framework for PfA is different from the reported structures of other L-asparaginases. The differences might be due to (i) a higher order multimeric structure engaging more active sites per molecule; (ii) spatial orientation of the active site restricting free access of the substrate, and (iii) tertiary/quaternary structural features surrounding the enzyme active site for intimate interaction with substrate.

The tetrameric EcAII is formed by simple dimer–dimer association; information about subunit association for the TthA hexamer is not available. Consequently, residues involved in multimerization and those contributing to catalysis probably are quite different from those found in *E. coli* or pyrococcal asparaginases. A sequence alignment of PfA with EcAI and EcAII showed 37 and 26% identity, respectively. The alignment also showed that out of eight active site residues, six were conserved (data not shown). However, when the sequence of PfA was aligned with TthA, no similarity was found. This is rather surprising because *Pyrococcus* being a thermophile (archaean) should be evolutionarily close to *T. thermophilus*. In contrast, the sequence comparison data suggests that PfA is closer to EcAI and EcAII. A homology-based model of PfA was obtained using Modeller 9v6 based on coordinates of PhA crystal structure (Fig. 4a; see color insert). Superposition of the PfA model on EcAII structure (Fig. 4b) showed a near perfect overlap with much of the active site residues falling spatially in close vicinity (Fig. 4c). Thus, both sequence and structural analysis support the evolutionary closeness between PfA and *E. coli* enzymes.

The difference in unfolding data in the presence of urea versus GdnCl is quite striking. Urea, even at 8 M concentration, could neither impose structural destabilization nor any activity loss. GdnCl however, caused unfolding and the data fitted well to a two-state model. Interestingly, at concentrations well below the beginning of unfolding transition there was a substantial activity loss. This motivated us to probe for subtle tertiary structural change in the enzyme active site. A difference in the near-UV-CD spectrum between the GdnCl-incubated and non-incubated protein samples indicated a distinct change in the neighborhood of aromatic amino acid residues (Fig. 4d). This might be because of Tyr21, a key residue in the enzyme active site. Tyr21 of PfA overlaps with Tyr25 of EcAII (Fig. 4c). In addition, a Tyr273 of PfA, placed in close vicinity of the active site, might also be perturbed at low denaturant concentration. Tyr25 of EcAII has been implicated as part of a flexible extended mobile loop for closing on the substrate for catalysis [41, 42]. A similar loop in the structure of PfA appears to be rather inflexible. This restricted mobility is possibly due to multiple H-bonding and ionic interactions of the loop residues with Lys274 at the enzyme active site (Fig. 4d). We propose that changing the Lys274 can release the rigid loop, thereby an active site with greater substrate accessibility might be created. This report serves as an important bridge supporting structural–functional relationship for thermophilic L-asparaginases.

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