***Thermotoga neapolitana* adenylate kinase is highly active at 30 °C**

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The adenylate kinase (AK) gene from *Thermotoga neapolitana*, a hyperthermophilic bacterium, was cloned and overexpressed in *Escherichia coli*, and the recombinant enzyme was biochemically characterized. The *T. neapolitana* AK (TNAK) sequence indicates that this enzyme belongs to the long bacterial AKs. TNAK contains the four cysteine residues that bind Zn²⁺ in all Gram-positive AKs and in a few other Zn²⁺-containing bacterial AKs. Atomic emission spectroscopy and titration data indicate a content of 1 mol of Zn²⁺/mol of recombinant TNAK. The EDTA-treated enzyme has a melting temperature ($T_m = 93.5$ °C) 6.2 °C below

that of the holoenzyme (99.7 °C), identifying Zn²⁺ as a stabilizing feature in TNAK. TNAK is a monomeric enzyme with a molecular mass of approx. 25 kDa. TNAK displays V_{max} and K_m values at 30 °C identical with those of the *E. coli* AK at 30 °C, and displays very high activity at 80 °C, with a specific activity above 8000 units/mg. The unusually high activity of TNAK at 30 °C makes it an interesting model to test the role of enzyme flexibility in activity.

Key words: hyperthermophilic enzyme, thermostability, zinc.

INTRODUCTION

Proteins from hyperthermophilic organisms are highly thermostable and are optimally active at high temperatures (often 90 °C and above). Most studies agree that stabilization mechanisms (such as hydrophobic interactions, hydrogen bonds and salt-bridges) vary from one protein to another and that no single molecular mechanism is responsible for protein thermal stabilization. One hypothesis explaining the remarkable thermostability of hyperthermophilic enzymes is that these enzymes have enhanced conformational rigidity at low temperatures. According to this hypothesis, psychrophilic, mesophilic, thermophilic and hyperthermophilic enzymes with sequence similarity have comparable catalytic efficiencies (indicated by k_{cat}/K_m values) at their respective optimal temperatures, because optimal activity requires a certain degree of conformational flexibility at the active site. Owing to their increased rigidity at low temperatures, thermophilic and hyperthermophilic enzymes are only marginally active at these temperatures, and they gain flexibility required for optimal activity only at higher temperatures [1]. Recent experimental results (e.g. amide hydrogen/deuterium exchange and Fourier-transform infrared spectroscopy) and molecular dynamics simulations show that results vary from one protein to another. Some thermophilic enzymes are less flexible than their mesophilic counterparts [2–4], whereas others are equally, if not more, flexible than their mesophilic counterparts [5]. The limited amount of experimental results and the type of data available (most of the experimental flexibility data are available only for the protein as a whole, rather than at the residue level) do not allow us to determine clearly whether rigidity at mesophilic temperatures is a key factor in protein thermostability. The time scale and amplitude of the conformational fluctuations that might lead to low thermostability are also not clear.

For these reasons, we decided to compare a new set of similar enzymes in terms of flexibility and thermostability. We chose the monomeric enzyme adenylate kinase (AK) because its small size (25 kDa) renders it accessible to NMR and molecular dynamics studies. AK (EC 2.7.4.3) catalyses the reaction $MgATP + AMP \rightarrow MgADP + ADP$. Because it is the only enzyme forming ADP from AMP, this ubiquitous enzyme is essential for the maintenance of the cellular ATP pool, for ATP production and for all macromolecular syntheses [6]. Over the past 27 years, numerous studies (e.g. X-ray crystallography, NMR spectroscopy and site-directed mutagenesis) have been performed to characterize the ATP- and AMP-binding sites of AK, to identify its catalytic mechanism and to understand the mechanisms underlying the conformational changes occurring during catalysis (reviewed in [7]).

Four AK families have been described previously [8,9]. The short AKs (family 1) represented by the mammalian cytosolic enzymes, and the long AKs (family 2), represented by the bacterial and mitochondrial enzymes differ by a 20–30-amino-acid residue insertion that is present in the long enzymes. This insertion creates a separate domain that acts as a lid covering the active site [10,11]. Among the bacterial enzymes, two subgroups of AKs can be differentiated by the presence or absence of a structural Zn²⁺ in the lid [11,12]. The four Zn²⁺ ligands (typically four cysteine residues or three cysteine residues plus a carboxylic residue) are conserved in all the Zn²⁺-containing AKs. [12]. The third AK family represented by methanococcal and *Sulfolobus acidocaldarius* AKs does not show significant similarity to the other two AK families, with the exception of the P loop, a signature motif in nucleotide kinases [9,13,14]. In contrast with the cytosolic and bacterial AKs, which are monomeric enzymes, methanococcal and sulfolobal AKs are trimeric [14]. A fourth AK subfamily was described recently and it accounts at least for the

Abbreviations used: AK, adenylate kinase; BSAK, *Bacillus stearothermophilus* AK; DSC, differential scanning calorimetry; ECAK, *Escherichia coli* AK; Ec-PRAI, *E. coli* phosphoribosyl anthranilate isomerase; GdnHCl, guanidine hydrochloride; ICP-AES, inductively coupled plasma-atomic emission spectroscopy; ORF, open reading frame; PAR, 4-(2-pyridylazo) resorcinol; PMPS, *p*-hydroxy-mercuriphenyl sulphonate; SB, superbroth; TMAK, *Thermotoga maritima* AK; Tm-PRAI, *T. maritima* phosphoribosyl anthranilate isomerase; TNAK, *T. neapolitana* AK.

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Mycobacterium capricolum enzyme [15]: this subfamily consists of short bacterial enzymes that are related to the long bacterial AKs more closely when compared with the short mammalian cytosolic AKs.

Despite the fact that monomeric AKs are well-studied small ubiquitous enzymes, and even though the *Escherichia coli* AK (ECAK) unfolds almost completely reversibly [16], monomeric AKs have rarely been studied in terms of thermostabilization mechanisms and in terms of their activity versus stability at high temperatures. Zhang et al. [17,18] noticed that inactivation of the muscle cytosolic AK always occurred at lower temperatures and at lower urea concentrations compared with other significant conformational changes. These authors suggested that the active site was more flexible than the rest of the enzyme. They also suggested that with increasing temperature the active site reached flexibility levels incompatible with catalysis, while the rest of the enzyme was still folded. The stabilization provided by Zn^{2+} in Zn^{2+} -containing AKs was studied by site-directed mutagenesis. A mutant ECAK engineered to contain a Zn^{2+} cation is significantly more stable than the wild-type enzyme [19]. This mutant ECAK is still less stable than the *Bacillus stearothermophilus* AK (BSAK), indicating that the Zn-finger-like structure in BSAK is not its sole stabilizing feature. This observation has been confirmed by two facts: (i) the *B. subtilis* AK, a Zn^{2+} -containing AK, is significantly less stable than BSAK, and (ii) it is not more stable than the wild-type ECAK [20].

No AKs have been characterized from hyperthermophilic bacteria. However, a wealth of AK structure information (e.g. X-ray and NMR data for ECAK and BSAK) is already available, which would facilitate comparative studies between mesophilic and hyperthermophilic bacterial AKs. For these reasons, we decided to characterize *Thermotoga neapolitana* AK (TNAK). In the present study, we show that, with a T_m close to 100 °C, TNAK is indeed a highly thermostable protein. The three enzymes ECAK, BSAK and TNAK thus represent a nice set of similar enzymes whose dynamic properties can be compared at the residue level using NMR and molecular dynamics approaches. With kinetic parameters identical with those of ECAK at 30 °C, TNAK is one of the rare hyperthermophilic enzymes that are highly active at mesophilic temperatures. These intriguing catalytic properties of TNAK will make the comparison of TNAK, ECAK and BSAK in terms of flexibility, activity and thermostability particularly interesting.

MATERIALS AND METHODS

Bacterial strains and plasmids

T. neapolitana strain 5068 ([21] and Deutsche Sammlung von Mikroorganismen und Zellkulturen catalogue, 1993) was used as the source of chromosomal DNA to construct the genomic library. *E. coli* strains XL1-Blue MRF' and XL0LR (Stratagene, La Jolla, CA, U.S.A.) were used as the host and the excision plating strains respectively for the *T. neapolitana* genomic library. *E. coli* strain CV2, which expresses the thermosensitive Pro⁸⁷ → Ser AK mutant [22], was used to express the *T. neapolitana* genomic library. Strain CV2 grows at 30 °C, but it cannot grow at 42 °C. Strain DH5 α (Life Technologies, Gaithersburg, MD, U.S.A.) was used for subcloning experiments. Strains HB101(DE3) and CV2(DE3) were built using the λ DE3 lysogenization kit (Novagen, Madison, WI, U.S.A.). Strain HB101(DE3) was used to overexpress the recombinant TNAK. *E. coli* strains were grown in Luria–Bertani (LB) or superbroth (SB) [23] medium, which contained 100 μ g/ml ampicillin or 25 μ g/ml kanamycin when necessary. Plasmid pCR2.1 (TA cloning kit; Invitrogen,

Carlsbad, CA, U.S.A.) was used to clone PCR products. Plasmid pET23a(+) (Novagen) was the expression vector.

Library construction and screening

T. neapolitana genomic DNA was extracted as described previously [24]. This genomic DNA was partially digested with *Sau*3A. *Sau*3A fragments (2–12 kb) were isolated on a 10–40 % sucrose gradient and cloned into the *Bam*HI/alkaline phosphatase-treated ZAP Express vector using the ZAP Express Predigested Gigapack II Gold Cloning kit (Stratagene). Mass excision of the library from the phage vector was performed according to the manufacturer's instructions. *E. coli* strain CV2 was transformed with the excised library and transformants were screened for growth at 42 °C.

Manipulation of DNA

Plasmid DNA purification, restriction analysis, subclonings and PCRs were performed using conventional techniques [25,26]. Oligonucleotides used for PCRs and for sequencing were synthesized by the Michigan State University Macromolecular Facility (East Lansing, MI, U.S.A.). DNA was recovered from agarose gels with the GeneClean II kit (BIO 101, La Jolla, CA, U.S.A.). DNA sequences were determined on both strands using the ThermoSequenase radiolabelled terminator cycle sequencing kit (U.S. Biochemical Corp., Cleveland, OH, U.S.A.). Sequencing data were analysed using version 8 of the Sequence Analysis Software Package of the Genetics Computer Group (University of Wisconsin, Madison, WI, U.S.A.) [27]. The GenBank[®] accession no. for the sequence published in the present paper is AF494055.

Subcloning of the AK gene from *T. neapolitana* into an expression vector

The AK gene from *T. neapolitana* (*adk*) was amplified using plasmid pTNAK1 as the template and oligonucleotides 5'-GCATATGATGGCTTATCTGGTGTTC-3' (where sequence CATATG creates an *Nde*I site) and 5'-CGAATTCCTTATCA-TTTATCACTCCACCC-3' (where sequence GAATTC creates an *Eco*RI site) as primers. The amplified *adk* gene was cloned into plasmid pCR2.1, and its sequence was verified. The *adk* gene was then subcloned into *Nde*I and *Eco*RI sites of the vector pET23a(+) to create plasmid pTNAK2. Since pTNAK2 was not well maintained in *E. coli*, the kanamycin-resistant Genblock (kan^R; Amersham Biosciences, Piscataway, NJ, U.S.A.) was cloned into the unique *Eco*RI site of pTNAK2. HB101(DE3)(pTNAK2::kan^R) cultures were grown in the presence of kanamycin.

AK purification

Fresh 4 ml HB101(DE3)(pTNAK2::kan^R) precultures in LB kanamycin were used to inoculate four 1 litre flasks of SB kanamycin, which contained 1 mM $ZnCl_2$. TNAK production was induced with 1 mM isopropyl- β -D-thiogalactoside when cultures reached an attenuation D_{600} of 1. After a 20 h induction, the cultures were centrifuged at 5000 *g* for 10 min and resuspended in 150 ml of 50 mM Tris/HCl, pH 7.4 (buffer A). The bacterial suspension was incubated at 37 °C for 1 h with 0.6 mg/ml lysozyme and a few mg of DNase I. Cells were then disrupted by two passages through a French pressure cell. The soluble and insoluble fractions were separated by centrifugation at 20 000 *g* for 20 min. The insoluble fraction was resuspended

in approx. 80 ml of buffer A. Both fractions were heat-treated at 80 °C for 20 min, cooled on ice for 5 min, and centrifuged again at 20 000 *g* for 20 min. Pellets containing heat-denatured proteins were discarded. Supernatants from the heat-treated soluble and insoluble fractions were pooled together, and they were loaded (at a flow rate of 0.8 ml/min) on to an Affi-Gel Blue Gel column (2.6 cm × 30 cm; Bio-Rad Laboratories, Hercules, CA, U.S.A.), pre-equilibrated with buffer A. After washing the column with 5 vol. of buffer A, proteins were eluted with a 500 ml linear 0–1 M NaCl gradient in buffer A. Fractions containing AK activity were pooled, dialysed against three batches of buffer A, then concentrated in an ultrafiltration cell equipped with a 10 kDa molecular mass cut-off membrane (Amicon, Beverly, MA, U.S.A.). The resulting 20 ml solution was loaded on to a Sephacryl-S100 HR column (2.4 cm × 90 cm; Pharmacia, Uppsala, Sweden), pre-equilibrated with buffer A. Elution was performed with buffer A at a flow rate of 50 ml/h. Fractions containing AK activity were pooled, dialysed against 20 mM Tris/HCl, pH 8.6 (buffer B), and concentrated by ultrafiltration as described above. This sample was loaded on to a DEAE-Sephacryl Fast Flow (Amersham Biosciences) anion-exchange column (2.6 cm × 30 cm; flow rate 1 ml/min) pre-equilibrated with buffer B. After washing the column with 5 vol. of buffer B, TNAK was eluted with a 500 ml linear 0–1.2 M NaCl gradient in buffer A.

Gel-permeation chromatography

The purified TNAK (0.8 mg in 0.3 ml of buffer A) was loaded on to a Sephadex G-75 column (1.0 cm × 40 cm). Elution was performed with buffer A at a flow rate of 0.2 ml/min. The molecular-mass markers used were from the Sigma kit for molecular masses in the range 65–66 kDa (catalogue no. MW-GF-70; Sigma, St. Louis, MO, U.S.A.).

AK assays

TNAK activity in the direction of ADP formation was determined by a coupled assay at 38 °C. The reaction mixture (1 ml) consisted of buffer A containing 250 mM KCl, 4 mM MgCl₂, 1 mM ATP, 1 mM AMP, 0.5 mM phosphoenolpyruvate, 0.25 mM NADH and 2.5 units each of pyruvate kinase (catalogue no. P-9136; Sigma) and lactate dehydrogenase (L-2500; Sigma). The reaction was started by the addition of TNAK, which was appropriately diluted with buffer A. NADH consumption was followed at 340 nm.

At temperatures above 40 °C, TNAK activity was determined by an end-point method. The reaction mixture (0.8 ml; buffer A containing 250 mM KCl, 4 mM MgCl₂, 1 mM ATP and 1 mM AMP) was incubated with TNAK for 2–5 min at the temperature of interest, and then cooled on ice. The reaction mixture (0.6 ml) was then transferred to 0.4 ml of buffer A, containing 250 mM KCl, 4 mM MgCl₂, 1.25 mM phosphoenolpyruvate, 0.625 mM NADH, 0.25 mM *P*¹,*P*⁵-di(adenosine-5')pentaphosphate ('Ap₅A') and 2.5 units of lactate dehydrogenase. After measuring the absorbance at 340 nm, 2.5 units of pyruvate kinase were added and absorbance was measured again.

To determine the kinetic parameters of TNAK, assays were performed in the presence of 0.05–2 mM ATP or 0.025–1 mM AMP, using the end-point method described above. One unit of enzyme corresponds to 1 μmol of product formed per min under the given conditions.

To determine the effect of pH on TNAK activity, enzyme activity was measured in 50 mM sodium acetate (pH 3.7–5.5), sodium phosphate (pK_{a2}) (pH 6.0–7.0), Tris (pH 6.5–8.0), glycine

(pH 8.5–10.5) and sodium phosphate (pK_{a3}) (pH 11.0–12.0) at 40 °C using the end-point method. All pH values were adjusted at room temperature (25 °C), and the Δ*pK_a*/Δ*t* for the different buffers [28] were considered to calculate the pH values at 40 °C. The assays were performed at a constant ionic strength of 220 mM. The amount of KCl added to adjust the ionic strength *I* was based on the equation $I = \sum (Z_i^2 [C_i])$, where *Z_i* is the number of charges of one of the ion and [*C_i*] its concentration.

Kinetic stability assays

The kinetic thermostability of TNAK was determined by incubating the enzyme (at protein concentrations of 0.55 mg/ml and 5.5 μg/ml) in 0.1 M sodium phosphate (pH 7.5) at 75, 80, 90, 95, 100, 105 and 110 °C for different time periods. The stability of TNAK at 5.5 μg/ml was also tested at 90 °C in the presence of 1 mg/ml BSA. After heat inactivation, samples were immediately cooled on ice, then diluted 1000 times with 0.1 M Tris (pH 7.4). TNAK residual activity was determined with 10 μl of these diluted samples using the coupled spectrophotometric assay. Remaining activity (100 %) corresponded to 1050 units/mg at 38 °C.

To determine the effect of pH on the kinetic stability of TNAK, TNAK (1.85 μg/ml) was incubated in 50 mM sodium acetate (pH 3.7–5.5), sodium phosphate (pK_{a2}) (pH 6.0–7.0), Tris (pH 6.5–8.0), glycine (pH 8.5–10.5) and sodium phosphate (pK_{a3}) (pH 11.0–12.0) [total ionic strength adjusted to 220 mM with KCl (calculated as above)] for 1 h at 50 °C. The residual activity of TNAK was assayed in 50 mM Tris (pH 7.4) using the coupled spectrophotometric method at 30 °C.

Microcalorimetry

Differential scanning calorimetry (DSC) was performed with an MC-2 microcalorimeter (Microcal, Northampton, MA, U.S.A.). Scans were performed with 2 mg/ml TNAK solutions in buffer A in the presence of different concentrations of high-purity guanidine hydrochloride (GdnHCl; Pierce, Rockford, IL, U.S.A.). The reference cell contained buffer A with the corresponding GdnHCl concentration. Thermal gradients were from 25 to 100 °C at a scanning rate of 1 °C/min. To minimize the background noise, a buffer–buffer scan was subtracted from the buffer–TNAK scan before data analysis. DSC scans of TNAK and the apoenzyme in the absence of the denaturant were performed at the Glasgow Biological Microcalorimetry Facility (University of Glasgow, Glasgow, U.K.). These scans were performed with 1.3 mg/ml TNAK in 50 mM Mops (pH 7.4), with a 1 °C/min thermal gradient from 25 to 125 °C.

Spectroscopic methods

Spectrophotometric titrations of Zn²⁺ in TNAK with *p*-hydroxymercuriphenyl sulphonate (PMPS) were performed in a Beckman DU650 spectrophotometer equipped with a Peltier system, using quartz microcuvettes (1 cm path length). The enzyme was extensively dialysed against 40 mM Hepes/NaOH (pH 7.2) (buffer C) before titration. Buffer C, PMPS and 4-(2-pyridylazo) resorcinol (PAR) solutions were prepared using MilliQ water. Release of Zn²⁺ from TNAK was followed after adding successive samples of 1–10 mM PMPS to 300 μl of 10 μM TNAK in buffer C, which contained 0.1 mM PAR. Absorbances at 250 and 500 nm were recorded 5 min after each PMPS addition. In each experiment, the first sample cuvette (10 μM TNAK in buffer

Table 1 Percentage identity (percentage similarity) of TNAK with selected prokaryotic AKs

Scores were obtained using the GAP software. Sequences were obtained from GenBank[®] and from genome sequences.

Enzyme	* <i>Archaeoglobus fulgidus</i> AK	<i>Aquifex aeolicus</i> AK	BSAK	ECAK	<i>Paracoccus denitrificans</i> AK	* <i>Pyrococcus abyssi</i> AK	* <i>Sulfolobus acidocaldarius</i> AK	* <i>Methanococcus igneus</i> AK
TNAK	60.7 (70.1)	48.5 (60.2)	44.7 (57.6)	46.7 (56.7)	41.1 (56.1)	37.7 (53.0)	31.5 (43.2)	21.4 (34.1)

* Archaeal species.

C plus 0.1 mM PAR) was used as the blank, so that absorbance values recorded thereafter were values of ΔA_{250} and ΔA_{500} . Initial values of A_{250} and A_{500} for other sample cuvettes were measured before adding the first PMPS sample. These initial absorbance values were later subtracted from the experimental readings to eliminate variations due to cuvette differences.

CD spectroscopy was performed on a JASCO J-170 spectropolarimeter (JASCO, Easton, MD, U.S.A.) equipped with a 1 ml quartz cylindrical cuvette. The sample solutions contained 0.03 mg/ml TNAK in 10 mM Tris (pH 7.4) in the presence of different concentrations of high-purity GdnHCl. Solutions were incubated overnight at room temperature for equilibration. For each GdnHCl concentration, a CD spectrum was recorded in the absence of the enzyme. This spectrum was subtracted from the enzyme spectrum before data were analysed.

Other analytical procedures

Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories) with BSA as the standard. SDS/PAGE was performed as described previously [29]. Proteins were visualized by Coomassie Blue staining. Elemental analysis was performed by inductively coupled plasma-atomic emission spectroscopy (ICP-AES) at the Animal Health Diagnostics Laboratory, Michigan State University. The dialysis buffer was used as the negative control for ICP-AES.

RESULTS

Cloning of the *adk* gene from *T. neapolitana* in *E. coli*

E. coli strain CV2 transformed with the *T. neapolitana* genomic phagemid library was plated on LB ampicillin agar and incubated at 42 °C. One colony was obtained after a 2 day incubation. The phagemid (pTNAK1) extracted from this colony contained a 3 kb insertion. Transformation of strain CV2 with pTNAK1 allowed CV2 to grow at 42 and 45 °C. The cell extract of CV2(pTNAK1) showed six times more AK activity than the cell extract of CV2 without the plasmid.

Nucleotide sequence of the *adk* gene from *T. neapolitana*

The pTNAK1 insert was sequenced using vector- and insert-specific primers. Two open reading frames, ORF1 and ORF2, were identified. ORF1 was truncated at its 5'-terminus. The two ORFs overlap by one nucleotide (results not shown). A BLAST search using the truncated peptide encoded by ORF1 as the probe showed significant similarity to SecY, a subunit of the SecAEGY preprotein translocase complex. A BLAST search using the peptide encoded by ORF2 as the probe showed significant similarity to multiple AKs. A potential ribosome-binding site was identified 4–7 nt upstream of ORF2 (i.e. the

adk gene). No potential promoter could be identified, suggesting that the *T. neapolitana adk* gene is co-transcribed with *secY*.

Sequence comparisons

As shown in Table 1 and Figure 1, TNAK belongs to the family of long bacterial AKs. With the exception of *Thermotoga maritima* AK (TMAK) (with which it is 93.2% identical), TNAK is more similar to the archaeal *Archaeoglobus fulgidus* AK than to other bacterial AKs (Table 1). TNAK also shows significant similarity to another archaeal AK, the *Pyrococcus abyssi* enzyme. These two archaeal AKs show much higher similarity to the long bacterial AKs, in general, than to the AKs from the archaea *S. acidocaldarius* and *Methanococcus igneus* (results not shown), suggesting that archaeal AKs do not represent a phylogenetically homogeneous enzyme group.

Most residues that bind ATP and AMP in ECAK [10] are conserved in TNAK as well as in the *A. fulgidus* and *P. abyssi* enzymes (Figure 1). The ATP- and AMP-binding residues of ECAK that are not conserved in TNAK correspond either to ECAK residues that have one atom at a 4.5 Å distance from ATP or AMP or to residues that interact with the nucleotides through their backbone nitrogen or oxygen atoms. The four Zn²⁺-liganding cysteine residues characterized in all AKs from Gram-positive bacteria [30] and in a few other bacterial AKs [31,32] are present in TNAK (Figure 1) and in TMAK (results not shown). These cysteine residues are also present in the *A. fulgidus* and *P. abyssi* enzymes (Figure 1).

TNAK overexpression in *E. coli* and purification

We first attempted to overexpress TNAK in strain BL21(DE3) (Stratagene). The expression level of TNAK remained mediocre (approx. 5 mg/l of culture) even after induction with isopropyl- β -D-thiogalactoside. When we tried expressing TNAK in strain CV2(DE3), no overexpression was observed with or without induction. *E. coli* strain HB101(DE3) gave satisfying results: TNAK was not expressed at significant levels in the absence of induction, and approx. 50 mg/l TNAK was obtained after 20 h induction in SB medium.

During TNAK purification, a significant fraction of the recombinant enzyme (approx. 30%) was present in the pellet after centrifugation of the bacterial crude extract. Most of this enzyme was recovered in solution after resuspension of the pellet in buffer A, heat treatment at 80 °C and centrifugation. The heat treatment at 80 °C was a highly efficient purification step, and it allowed us to obtain the enzyme preparation without any contaminating ECAK that was initially present in the crude extract. Our purification procedure yielded pure TNAK, as determined by SDS/PAGE (results not shown). The purified TNAK consisted of a single subunit of 25 kDa (as determined by SDS/PAGE), and it migrated as a 29 kDa protein during gel permeation on Sephadex G-75, indicating that TNAK is a monomeric enzyme, a trait typical of

Figure 1 Alignment of *T. neapolitana* (Tnea), *A. fulgidus* (Aful), *B. stearotherophilus* (Bste), *A. aeolicus* (Aaeo), *E. coli* (Ecol) and *P. abyssi* (Paby) AK sequences

Grey background and framed residues indicate residues interacting with ATP and AMP respectively [10]. ↓, residues involved in Mg²⁺ binding (through water molecules) [11]; *, cysteine residues involved in Zn²⁺ binding.

long bacterial AKs. The purified TNAK showed a specific activity of 1050 units/mg enzyme at 38 °C.

Effects of temperature and pH on TNAK activity and stability

The specific activity of TNAK, as determined by the end-point method, increased with temperature to its maximum (8400 units/mg) at 80 °C, and then it started to decrease sharply (Figure 2A). The Arrhenius plot of TNAK activity (ADP formation) against temperature was linear between 30 and 70 °C (Figure 2B), with activation energy of 49 kJ/mol. This value is of the same order of magnitude as the activation energies for the reactions catalysed by the muscle cytosolic AK (38 kJ/mol) [17], by ECAK (46 kJ/mol) and by BSAK (73 kJ/mol) [30]. The kinetic thermostability of TNAK was tested at two different enzyme concentrations (Figures 2C and 2D). TNAK is significantly more stable at high concentrations or in the presence of BSA than at low concentrations. At both concentrations tested, inactivation is of first order, with activation energies of 185.6 kJ/mol at 0.55 mg/ml enzyme and 44.4 kJ/mol at 5.5 µg/ml enzyme. TNAK is optimally active between pH 7.4 and 8.0. It is more than 70% active at pH 7.0 and 8.5 (Figure 3A). The enzyme is optimally stable at pH 8.3 and retains 80% activity at pH 7.4 and 9.3. TNAK is not at all stable at pH values below 5.0 (Figure 3B).

TNAK stability studied by calorimetry and CD spectroscopy

We did not detect any obvious melting transitions in our DSC experiments with TNAK in buffer A at temperatures below 100 °C (the upper temperature limit for our MC-2 microcalorimeter). For this reason, we followed TNAK thermal unfolding in the presence of GdnHCl. Melting was almost completely reversible at a GdnHCl concentration of 0.4 M, as indicated by the almost complete superimposability of the excess heat capacity curves obtained in two consecutive scans (results not shown). The percentage of refolding after cooling decreased with increasing GdnHCl concentration. At 2.6 M GdnHCl, almost no transition was seen during a second scan. For this reason, no thermodynamic

Figure 2 Effect of temperature on TNAK activity and stability

(A) Specific activities of TNAK (□), ECAK (◆), *B. subtilis* AK (▲) and BSAK (■) at various temperatures [20,30]. (B) Arrhenius plot of TNAK activity. (C, D) TNAK concentrations were 0.55 mg/ml (C) and 5.5 µg/ml (D). (C) □, 90 °C; ○, 100 °C; △, 105 °C; ▨, 110 °C; (D) □, 75 °C; ●, 90 °C + BSA (BSA concentration = 1 mg/ml); ◇, 80 °C; ○, 90 °C; △, 95 °C.

analysis of the data was possible. In Figure 4(A), T_m of TNAK is shown as a function of GdnHCl concentration. A linear curve fit of the data ($y = -10.216x + 99.334$ with $r^2 = 0.991$) would predict a T_m of 99.3 °C in the absence of the denaturant. A second-degree polynomial fit of the data ($y = -1.315x^2 - 6.231x + 97.125$ with $r^2 = 0.996$) would predict a T_m of 97.1 °C in the absence of the denaturant. A confirmatory DSC scan of TNAK in the absence of the denaturant was performed by the Glasgow Biological Microcalorimetry Facility on a calorimeter

Figure 3 Effect of pH on TNAK activity (A) and stability (B)

Buffers used: ○, sodium acetate; △, sodium phosphate (pK_{a2}); ◇, Tris; □, glycine; ●, sodium phosphate (pK_{a3}).

Figure 4 TNAK unfolding in the presence of GdnHCl

(A) T_m as a function of GdnHCl concentration, followed by DSC. TNAK concentration was 2 mg/ml (□). For T_m in the absence of GdnHCl, the TNAK concentration was 1.3 mg/ml (■). The two lines represent the linear curve fit and the second-degree polynomial fit of the data (see the Results section). (B) TNAK (30 μ g/ml) equilibrium unfolding induced by GdnHCl at 25 °C as determined by the ellipticity value at 222 nm.

that allowed thermal gradients to be run up to 125 °C. At a scan rate of 1 °C/min, TNAK (1.3 mg/ml) showed a T_m of 99.7 °C. The melting transition observed in the first scan was not observed in the second scan, indicating that under these conditions, TNAK unfolded irreversibly. This T_m value is significantly above the values measured for ECAK, *B. subtilis* AK and BSAK (Table 2) [20,30]. It also corresponds to the T_m predicted by linear extrapolation of the melting data obtained in the presence of GdnHCl.

The TNAK GdnHCl equilibrium unfolding profile obtained by CD spectroscopy at 25 °C is similar to that of other long bacterial AKs, with the following exception: GdnHCl concentration at the midpoint transition for TNAK (i.e. 3.1 M) is much higher than that for ECAK, *B. subtilis* AK and BSAK (0.98, 1.38 and 2.45 M respectively) [20,30].

Table 2 T_m values of ECAK [19], *B. subtilis* AK, BSAK [30] and TNAK

Enzyme	T_m (°C)	Reference
ECAK	51.8	[19]
ECAK quadruple Cys mutant containing Zn^{2+}	63.0	[19]
<i>B. subtilis</i> AK	50.7	[20]
BSAK	74.5	[30]
Apo-BSAK	67.0	[30]
TNAK	99.7	This study
Apo-TNAK	93.5	This study

Table 3 Kinetic parameters of ECAK, BSAK and TNAK

Enzyme	Temperature (°C)	K_m		V_{max} (ATP, AMP) (units/mg of protein)*	Reference
		ATP (μ M)	AMP (μ M)		
ECAK	30	51	38	1020	[30]
BSAK	37	36	76	288	[30]
TNAK	30	49.4 ± 4.9	40.9 ± 6.6	1130	This study

* No S.D. is available for V_{max} (ATP, AMP), because it is the mean V_{max} value calculated from V_{max} (ATP) and V_{max} (AMP).

Table 4 Zn^{2+} content in TNAK as determined by ICP-AES

Treatment	Temperature (°C)	Duration (min)	Zn^{2+} content in TNAK (mol/mol)	Remaining activity (%)
None	—	—	0.88	100
EDTA (10 mM)	20	30	0.85	n.d.*
		40	0.91	n.d.
		60	0.76	n.d.
		70	0.58	n.d.
		75	0.24	n.d.
		80	0.23	94.6
Phenanthroline (10 mM)	75	60	0.58	n.d.
GdnHCl (5 M) + EDTA (10 mM)	20	60	0.26	n.d.

* n.d., not determined.

Kinetic properties of TNAK

Because TNAK activity at temperatures between 30 and 45 °C is comparable with that of ECAK (Figure 2A), the kinetic parameters of TNAK were determined at 30 °C for comparison with those of ECAK. As shown in Table 3, the kinetic parameters of TNAK in the direction of ADP formation were more or less identical with those of ECAK at the same temperature, namely 30 °C.

Determination of the Zn^{2+} content of TNAK

The four cysteine residues involved in Zn^{2+} binding in other AKs [30] are present in TNAK also. This suggests that TNAK also contains Zn^{2+} . The presence of Zn^{2+} in TNAK was tested by ICP-AES and by titration with the strongly dissociating thiol-specific reagent PMPS. ICP-AES results (Table 4) indicate that the recombinant TNAK contains 1 mol of Zn^{2+} /mol of enzyme. Treatments with 10 mM EDTA for 30 min at 60 °C were almost completely inefficient in removing Zn^{2+} from TNAK. EDTA treatment for 1 h at 75 or 80 °C was necessary to deplete approx. 75 % Zn^{2+} from TNAK (Table 4). Other treatments (i.e. with

Figure 5 Titration of Zn^{2+} in TNAK with PMPS

□, ΔA_{250} (ΔOD_{250}); ○, ΔA_{500} (ΔOD_{500}). [PMPS] and [TNAK] were corrected for dilution before calculating the [PMPS]/[TNAK] ratios. ΔA_{250} and ΔA_{500} values were corrected for dilution.

10 mM phenanthroline or with 5 M GdnHCl plus 10 mM EDTA) were not more efficient than the EDTA treatments. As has been observed with BSAK [30], reducing the Zn^{2+} content of TNAK does not affect its activity (Table 4).

We confirmed the ICP-AES results by titrating Zn^{2+} in TNAK with PMPS at 25 °C. Formation of the PMPS–thiol chromophore (followed at 250 nm; Figure 5) shows that PMPS is linearly incorporated into TNAK, up to approx. 4 mol of PMPS/mol of TNAK. ΔA_{250} becomes constant once the ratio [PMPS]/[TNAK] > 4. PMPS binding is accompanied by the release of Zn^{2+} , which immediately forms $(\text{PAR})_2\text{Zn}^{2+}$ complexes that can be detected at 500 nm. Parallel with what is observed at 250 nm, Zn^{2+} is linearly released from TNAK up to approx. 4 mol of PMPS/mol of TNAK (followed at 500 nm; Figure 5). With a molar absorption coefficient of $6.6 \times 10^4 \text{ M}^{-1} \cdot \text{min}^{-1}$ for $(\text{PAR})_2\text{Zn}^{2+}$ at 20 °C [33], a complete release of Zn^{2+} from TNAK (i.e. $6.58 \mu\text{M}$) would give a ΔA_{500} of 0.43. The value of ΔA_{500} reached at the plateau (i.e. 0.42) corresponded to 0.98 mol of Zn^{2+} /mol of enzyme, a result that agrees with the ICP-AES results.

Thermostability of the apo-TNAK

A quadruple cysteine mutant ECAK capable of binding Zn^{2+} had a T_m 11 °C above that of the wild-type ECAK [18]. The apo-BSAK had a T_m 7.5 °C lower than that of the holo-BSAK [26]. These two results indicate that Zn^{2+} binding is a stabilizing feature in these two AKs. To test the role of Zn^{2+} binding in the thermodynamic stability of TNAK, we measured the T_m of apo-TNAK by DSC. To prepare the apoenzyme, TNAK was incubated at 80 °C for 1 h in the presence of 10 mM EDTA. It was then extensively dialysed against buffer A containing 2 mM EDTA and also against three batches of metal-free buffer A. By DSC, the apoenzyme shows a T_m approx. 6 °C below that of the holoenzyme (Table 2).

DISCUSSION

As expected from TNAK sequence [32], TNAK belongs to the long bacterial AKs. Since some proteins that are known to be monomeric in mesophiles exist as multimers in hyperthermophiles [34–36], and since some AKs are trimeric [14], it was important for us to determine the quaternary structure of TNAK. The fact that TNAK is a monomeric enzyme that is highly similar to ECAK and BSAK (Table 1, Figure 1) makes ECAK,

BSAK and TNAK a perfect set of similar enzymes whose dynamic properties can be compared at the residue level to correlate thermostability and activity with conformational dynamics.

Molecular mechanisms underlying the thermostability of TNAK are unknown. TNAK contains a significantly smaller number of alanine and glycine residues when compared with ECAK or BSAK (26 in TNAK versus 39 in ECAK and 40 in BSAK). Six alanine and glycine residues in ECAK are substituted with charged residues in TNAK. These six residues are located on the surface of ECAK [as visualized in the X-ray structure of ECAK (PDB code 1ake); results not shown]. Of the seven bulky hydrophobic residues of ECAK (i.e. Ile, Leu, Met or Val) substituted with charged residues in TNAK, six are exposed to the solvent (results not shown). Although these and other similar, not mentioned, observations suggest that TNAK might be stabilized by optimized hydrophobic packing and by an increased number of surface salt bridges, these stabilization mechanisms remain highly hypothetical in the absence of supporting structural and experimental evidence (the crystallization of TNAK is in progress).

The similarity of TNAK to the archaeal *A. fulgidus* AK is an intriguing result. Such a high similarity level suggests that the two genes originate from a common ancestor. Although evidence exists suggesting that lateral gene transfer has occurred between thermophilic bacteria and archaea [37], the *adk* gene might not be among the genes that have been laterally transferred. In *A. fulgidus*, the *adk* gene is not preceded by *secY* [38], as it is in *T. neapolitana*. In addition, the *T. maritima* *adk* gene does not belong to one of the 15 genomic cluster regions that are most archaea-like [37].

As shown in Figure 2(A) and Table 3, TNAK is as active as mesophilic and thermophilic bacterial AKs between 30 and 65 °C. In particular, it is three times as active at 30 °C as BSAK is at 37 °C. This result is highly uncommon. Only three other hyperthermophilic enzymes have been characterized that are highly active at mesophilic temperatures: (i) the *T. maritima* L-isoaspartyl O-methyltransferase [39] is optimally active at 85 °C, with a specific activity that is at least ten times higher than that of mesophilic enzymes at 25–37 °C. When compared with similar mesophilic enzymes, the *T. maritima* L-isoaspartyl O-methyltransferase had the lowest K_m values for each of the methyl-accepting substrates examined at 37 °C. (ii) The *T. maritima* indoleglycerol phosphate synthase [40] is twice as efficient as the *E. coli* enzyme at 25 °C. This higher catalytic efficiency for the hyperthermophilic enzyme is due to a 70 times higher affinity for the substrate, which compensates for a 33 times lower k_{cat} value. (iii) The *T. maritima* phosphoribosyl anthranilate isomerase (Tm-PRAI) [41] is almost four times more efficient at 25 °C than its *E. coli* counterpart, *E. coli* phosphoribosyl anthranilate isomerase (Ec-PRAI). Although the k_{cat} value of Tm-PRAI is almost ten times lower than that of Ec-PRAI, the affinity of Tm-PRAI for its substrate is almost 44 times higher than that of Ec-PRAI. For these three *T. maritima* enzymes, the enzyme activity is increased due to a very high affinity for their substrate. Their k_{cat} , however, remains typically one order of magnitude below that of the mesophilic protein. In TNAK, both K_m and k_{cat} values are identical with those of ECAK at 30 °C, making TNAK, again, a unique hyperthermophilic enzyme.

Hyperthermophilic enzymes are usually less active than their mesophilic counterparts at low temperatures. One explanation is that hyperthermophilic enzymes are too rigid at low temperatures to be highly active. Their flexibility increases with temperature, and reaches levels compatible with optimal activity only at temperatures close to the growth temperature of their source organism. This hypothesis and the fact that TNAK shows kinetic

parameters at 30 °C identical with those of ECAK suggest that the conformational flexibility of the active site of TNAK should be comparable with that of the active site of ECAK at 30 °C. The small size of bacterial AKs (20–25 kDa) makes possible the use of techniques such as NMR relaxation studies or hydrogen/deuterium exchange coupled with NMR detection, to study flexibility at the atomic level. These techniques will be used to compare the molecular dynamics of TNAK and ECAK catalytic sites at 30 °C.

The facts that TNAK at 0.55 mg/ml is kinetically and thermodynamically stable at 90 °C and that the optimal temperature for TNAK activity is only 80 °C suggest at first that TNAK reversibly inactivates between 80 and 90 °C. On the other hand, TNAK enzymic assays are typically performed using TNAK concentrations below 1 µg/ml. These concentrations are lower than the concentrations used for the kinetic thermostability assays or for DSC. The lower kinetic thermostability of TNAK at low concentrations suggests that the decrease in its activity at temperatures above 80 °C is due to an irreversible inactivation. This result is different from what Zhang et al. [17] concluded for the muscle cytosolic AK. These authors suggested that the active site was more flexible than the rest of the enzyme, and that the active site reached flexibility levels incompatible with catalysis with increasing temperature, while the rest of the enzyme was still folded.

We observed a linear relationship between T_m and GdnHCl concentration (Figure 4A). It is interesting to note that the T_m values obtained in the absence of GdnHCl correspond to the T_m values predicted by extrapolation from the melting data obtained in the presence of GdnHCl. Although we cannot, at this time, explain this correlation thermodynamically, we observed similar linear relationships between T_m values and GdnHCl concentration for the *Thermoanaerobacter ethanolicus* secondary alcohol dehydrogenase [42] and for the *T. neapolitana* xylose isomerase (V. Tchernajenko, C. Vieille and J.G. Zeikus, unpublished work). It is not surprising to observe a higher GdnHCl concentration for the midpoint of the unfolding transition of TNAK than for ECAK and BSAK. Although an increase in GdnHCl concentration at the midpoint of the unfolding transition of the enzyme is not an absolute rule for hyperthermophilic proteins, similar observations have been made for many of them [43,44].

TNAK is kinetically unstable at pH values below 5.0. This result is different from that observed with the *E. coli* and chicken AKs, which are stable under acidic conditions. This property has been used for their purification: a crude cell extract is acidified, neutralized, and then denatured proteins are removed by centrifugation [17,45,46]. It would be interesting to determine whether the acid-instability behaviour of TNAK is due to its thermostability requirements.

The native *Paracoccus denitrificans* AK and TMAK apparently do not contain any Zn^{2+} [12]. To determine whether these two enzymes contained any Zn^{2+} , *P. denitrificans* and *T. maritima* were grown in the presence of $^{65}ZnCl_2$; the AKs were purified from the bacterial crude extracts and tested for radioactivity [12]. At no point have these two enzymes been tested for the presence of a metal other than Zn^{2+} . Since the recombinant *P. denitrificans* AK expressed in *E. coli* contains an equal amount of Fe^{2+} and Zn^{2+} [31], we cannot exclude the possibility that the native *P. denitrificans* AK contains Fe^{2+} rather than Zn^{2+} . The same possibility exists for the native TMAK. Purification and characterization of these two native enzymes would be necessary to determine whether they contain any metal at all. In any case, with a T_m of 93.5 °C, the apo-TNAK remains stable and active at the temperatures compatible with *T. neapolitana* growth (i.e.

75–80 °C). Thus although Zn^{2+} stabilizes TNAK, its presence in the enzyme is not absolutely required for TNAK's function *in vivo*.

As observed with *Pyrococcus furiosus* α -amylase [47], removing metals from hyperthermophilic enzymes often requires harsh treatments. Here, a 1 h EDTA treatment at 80 °C is not totally efficient in removing Zn^{2+} from TNAK. But in contrast with *P. furiosus* α -amylase, the Zn^{2+} -liganding cysteine residues of TNAK are accessible for equimolar reaction with PMPS at 25 °C.

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