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An extremely thermostable aromatic aminotransferase from the hyperthermophilic archaeon *Pyrococcus furiosus*

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Abstract

Pyrococcus furiosus is a strictly anaerobic archaeon (formerly *archaebacterium*) that grows optimally at 100°C by the fermentation of peptides. Cell-free extracts were found to contain two distinct aromatic aminotransferases (ArAT, EC 2.6.1.57), one of which was purified to electrophoretic homogeneity. *P. furiosus* ArAT is a homodimer with a subunit M_r value of 44 000 ± 1000. Using 2-ketoglutarate as the amino acceptor, the purified enzyme catalyzed the pyridoxal 5′-phosphate (PM*P*)-dependent transamination of phenylalanine, tyrosine and tryptophan with respective k_{cat} values of 253, 72 and 62 (s^{-1} at 80°C) under saturating conditions. The K_m values for all three amino acids were between 1.1 and 2.1 mM and the optimum temperature for catalysis was above 95°C. The melting point for the pure enzyme was also above 95°C as determined by the change in ellipticity at 220 nm. Irreversible denaturation of the pure enzyme was not apparent after 6 h at 80°C in the presence of PM*P* and 2-ketoglutarate and the time required for a 50% loss in activity at 95°C was approx. 16 h. This decreased to approx. 12 h if cofactor and substrate were not added. In contrast, the apoenzyme (lacking PM*P*) lost most (70%) of its activity (measured after reconstitution) after 6 h at 80°C, indicating that both PM*P* and 2-ketoglutarate stabilize the enzyme at extreme temperatures. Although few ArATs have been characterized to date, the molecular properties and substrate specificity of *P. furiosus* ArAT more resemble those of the ArAT from *Escherichia coli* than those of the analogous enzyme from rat liver. Moreover, the *P. furiosus* enzyme is by far the most thermostable aminotransferase of any type to be purified so far.

Keywords: Aromatic aminotransferase; Hyperthermophile; Enzyme purification; Enzyme characterization; Archaeon; Anaerobic bacterium; *(P. furiosus)*

1. Introduction

Aromatic aminotransferases (ArAT, EC 2.6.1.57) are pyridoxal 5′-phosphate-dependent enzymes which catalyze the reversible transfer of the amino group from aromatic amino acids to 2-keto acids. ArATs are widely distributed in both eukaryotes and prokaryotes, but they have not been as thoroughly characterized as the closely related aspartate aminotransferases (AspAT, EC 2.6.1.1). An ArAT exhibiting high activity with tyrosine has been purified from *Escherichia coli* and is known as TyrAT*Ec* (EC 2.6.1.5) [1]. The biosynthesis of this enzyme depends on the transcription of *tyrB* and is repressed by tyrosine [2,3]. On the other hand, the biosynthesis of aspartate aminotransferase (AspAT*Ec*), which is most active with aspartic acid [1], depends on the transcription of *aspC* and is not affected by the presence of tyrosine [2,3]. Other than *E. coli*, aminotransferases specific for aromatic amino acids have been characterized in only one other prokaryote, from the methanogenic archaeon (formerly *archaebacterium*) *Methanococcus aeolicus* [4]. This organism contains two distinct enzymes, termed ArAT-I*Ma* and ArAT-II*Ma*, which were partially purified. They differed from each other in their molecular properties but their metabolic and physiological relationships are unknown [4].

Abbreviations: ArAT, aromatic aminotransferase; ArAT*Pf*, aromatic aminotransferase from *Pyrococcus furiosus*; ArAT-I*Ma*, aromatic aminotransferase I from *Methanococcus aeolicus*; ArAT-II*Ma*, aromatic aminotransferase II from *Methanococcus aeolicus*; AspAT, aspartate aminotransferase; AspAT*Ec*, aspartate aminotransferase from *Escherichia coli*; AspAT*Ss*, aspartate aminotransferase from *Sulfolobus solfataricus*; PM*P*, pyridoxamine 5′-phosphate; TyrAT, tyrosine aminotransferase; TyrAT*Ec*, tyrosine aminotransferase from *Escherichia coli*.

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Whereas the prokaryotic ArATs characterized so far exhibit significant transaminase activity with all three of the aromatic amino acids, there is an enzyme in mammals termed tyrosine aminotransferase (TyrAT) which specifically catalyzes the transamination of tyrosine [5]. TyrAT has attracted a lot of interest because its biosynthesis is hormonally regulated [6]. Although rat liver contains very low amounts of this enzyme, its gene was recently isolated and expressed in *E. coli* which led to the clarification of its catalytic and molecular properties [7,8].

This paper describes the purification of ArAT from the non-methanogenic archaeon, *Pyrococcus furiosus*. *P. furiosus* is a strict anaerobe which grows optimally at 100°C [9]. It belongs to the restricted class of organisms that grow near and above 100°C known as hyperthermophiles, virtually all of which are classified as Archaea [10–12]. Most of these organisms are obligate heterotrophs that utilize peptides or protein as their sole carbon and nitrogen source. A few, including *P. furiosus*, are able to use a limited number of carbohydrates as an additional carbon source, and the pathway of sugar oxidation has been elucidated (for review, see [12]). However, although active proteinases have been found in many of the heterotrophic hyperthermophiles (see [12]), the metabolism of amino acids by these organisms has not been investigated. The molecular and catalytic properties of *P. furiosus* ArAT (ArAT*Pf*) reported herein are discussed both in terms of the known properties of the ArATs from mesophiles and on the nature of the extreme stability of enzymes isolated from hyperthermophilic organisms [12].

2. Materials and methods

2.1. Materials

Q-Sepharose Fast Flow, S-Sepharose Fast Flow, Phenyl-Sepharose HiLoad (35/100) and Superdex-200 HiLoad (16/60) were purchased from LKB-Pharmacia. All separations on conventional and FPLC columns were performed using a Pharmacia-LKB FPLC system. Amino acids, 2-ketoglutarate and pyridoxamine 5′-phosphate were obtained from Sigma.

2.2. Aminotransferase assays

Aromatic aminotransferase activity was measured by a modification of the method described by George et al. [16]. The assay is based on the arsenate-catalyzed formation of aromatic 2-ketoacid-*enol*-borate complexes which show characteristic absorption spectra in the near-UV region. The assays were carried out in 0.2 M potassium phosphate buffer (pH 7.6), containing 9.4 mM 2-ketoglutarate and 62.5 μM PM*P*. The amino acid substrate concentration was 19.5 mM in the case of phenylalanine and tryptophan and 9.75 mM in the case of tyrosine. The assay mixture was incubated at 80°C and the enzyme was added to initiate the reaction. After a 5 min incubation at 80°C, the reaction was stopped by the addition of 20% (w/v) trichloroacetic acid to a final concentration of 4% (w/v) and the mixture was rapidly cooled in ice. After centrifugation, the supernatant was diluted 1:6 with a solution containing 1.0 M sodium arsenate and 1.0 M boric acid (pH 6.5) and was incubated for 25 min at 23°C. The amount of aromatic *enol*-borate complex produced was estimated using the molar extinction coefficient of 7100 $M^{-1}cm^{-1}$ at 300 nm for phenylpyruvate-*enol*-borate, 10700 $M^{-1}cm^{-1}$ at 310 nm for *p*-hydroxyphenylpyruvate-*enol*-borate, and 10800 $M^{-1}cm^{-1}$ at 330 nm for indolepyruvate-*enol*-borate [16]. One unit of enzyme activity is the amount which catalyzes the conversion of 1 μmol substrate per minute under these conditions. The assay described above was used to measure the activity of ArAT*Pf* at different temperatures ranging from 30°C to 95°C. The kinetic parameters for phenylalanine, tryptophan and tyrosine were determined at 80°C using the same procedure, but varying the amino-acid concentrations in the assay mixture.

2.3. Purification procedure

P. furiosus (DSM 3638) was grown as described previously [13]. ArAT*Pf* was purified using 500 g of cells (wet weight) as the starting material. All the purification steps were performed at room temperature. To maintain the enzyme in the pyridoxal form, pyridoxamine 5′-phosphate (PM*P*: 0.1 mM) and 2-ketoglutarate (2 mM) were added to all chromatography fractions containing ArAT activity after each purification step. ArAT was obtained from the same batch of *P. furiosus* cells routinely used to purify a variety of oxygen-sensitive oxidoreductase-type enzymes, e.g. [12–15]. Hence, the preparation of the cell-free extract and the first chromatography step were carried out under anaerobic conditions. All buffers were repeatedly degassed and flushed with Ar before use, and they were maintained under a positive pressure of Ar (see [13]).

Frozen cells were resuspended in 50 mM Tris-HCl buffer (pH 8.0), containing 2 mM sodium dithionite to protect against trace O_2 contamination. Lysozyme and DNase were added to obtain final concentrations of 0.5 mg/ml and 0.5 μg/ml, respectively. Lysis was induced by incubating of the cell suspension under continuous stirring at 37°C for 1.5 h [13]. The supernatant fraction from the cell-free extract, obtained by centrifugation (25000 × *g* for 1 h), was applied to a column (7.5 × 20 cm) of Q-Sepharose Fast Flow equilibrated with 50 mM Tris-HCl (pH 8.5), containing 2 mM sodium dithionite and 2 mM dithiothreitol. Before loading, the sample was diluted 3-fold with the equilibration buffer. The column was washed with equilibration buffer (1000 ml), and a linear gradient (8500 ml) from 0 to 500 mM NaCl was applied at 8.5 ml/min. Fractions of 85 ml were collected.

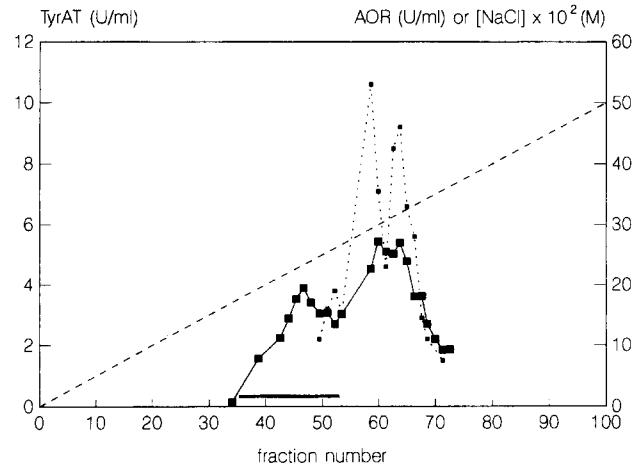

Fig. 1. Elution profiles of *P. furiosus* aromatic aminotransferase and aldehyde ferredoxin oxidoreductase from a Q-Sepharose Fast Flow column. A cell extract from *P. furiosus* was fractionated on a Q-Sepharose Fast Flow column equilibrated with 50 mM Tris-HCl (pH 8.5), containing 2 mM sodium dithionite and 2 mM dithiothreitol. Fractions were assayed for tyrosine aminotransferase (large square symbols) and aldehyde ferredoxin oxidoreductase [20] (small square symbols). The salt gradient used (---) and the fractions pooled for the next step (—) are indicated.

Subsequent purification steps were performed under aerobic conditions. The active fractions corresponding to the first peak eluted from Q-Sepharose (Fig. 1) were combined, concentrated by ultrafiltration (Amicon PM-10) to a final protein concentration of 14.7 mg/ml, and dialyzed against 50 mM Tris-HCl (pH 8.0) (buffer A). Ammonium sulfate was added to reach a final concentration of 1.8 M. After stirring overnight at 5°C, the sample was centrifuged (25 000 × *g* for 30 min) and the clear supernatant was loaded onto a Phenyl-Sepharose HiLoad column (35/100), equilibrated with buffer A containing 1.8 M ammonium sulfate, at a flow rate of 3 ml/min. After washing with two column volumes of the equilibration buffer, a linear gradient (975 ml) from 1.8 M to 0 M ammonium sulfate was applied at 7.5 ml/min. The active fractions were pooled, dialyzed against 50 mM sodium acetate (pH 5.0) (buffer B), and loaded at 3 ml/min onto a column (2.5 × 9 cm) of S-Sepharose Fast Flow equilibrated with buffer B. The column was washed with one volume of buffer B, and a linear gradient of NaCl (0–500 mM, 270 ml) in the equilibration buffer was applied at 3 ml/min. The active fractions that were judged electrophoretically pure, were pooled, dialyzed against buffer A, concentrated by ultrafiltration (Amicon PM-10) and stored at −20°C.

2.4. Miscellaneous

To obtain the apoenzyme form of ArAT, the holoenzyme was prepared in 2.0 M sodium acetate (pH 4.2), by ultrafiltration (Amicon PM-10), and was then precipitated by the addition of solid ammonium sulfate to 75% saturation. The precipitate was collected by centrifugation, dissolved in a minimal volume of 50 mM Tris-HCl (pH 8.0), and desalted on a PD-10 column (LKB-Pharmacia) equilibrated with the same buffer. The apoenzyme was assumed to be free of PM*P* as it exhibited no activity in the standard assay in the absence of added PM*P*. The holoenzyme was reconstituted by incubating the apoprotein for 1 h at 60°C in the presence of PM*P* (0.5 mM) and 2-ketoglutarate (0.025 mM). The reconstituted form exhibited the same specific activity in the standard assay as the native enzyme.

Protein concentrations were routinely estimated using the Bio-Rad Protein Assay System [17] with bovine serum albumin as the standard. Polyacrylamide gel electrophoresis was performed as described by Weber et al. [18] and proteins were detected by silver staining [19]. Aldehyde ferredoxin oxidoreductase was assayed by the crotonaldehyde-dependent reduction of methyl viologen at 80°C as previously described [20]. CD measurements were performed using a Jasco J-500A spectropolarimeter using cells with a 1 mm path length. ArAT*Pf* (0.54 mg/ml in 50 mM Tris-HCl, pH 8.0) was heated from 25 to 95°C over a period of 20 min and the ellipticity at 220 nm was continuously recorded. N-terminal sequence of the purified protein was determined by automated Edman degradation using an Applied Biosystem Sequencer fitted with an on-line phenylthiohydantoin amino-acid analyzer.

Table 1
Purification of aromatic aminotransferase from *P. furiosus*

Purification step	Volume (ml)	Protein (mg)	Phenylalanine amino-transferase activity			Tyrosine amino-transferase activity		
			(U)	(U/ml)	(U/mg)	(U)	(U/ml)	(U/mg)
Cell-free extract	1,300	32 500	59 000	45.3	1.8	36 725	28.3	1.13
Q-Sepharose [a]	1,445	3,084	13 566	64.6	4.4	4,318	20.6	1.4
Ammonium sulfate [b]	220	2,365	13 398	60.9	5.7	3,784	17.2	1.6
Phenyl-Sepharose	274	786	8713	31.8	11.1	2,751	10.0	3.5
S-Sepharose	60	39.9	7254	121.0	181.8	2,378	39.6	59.6

[a] These values correspond only to the first peak of aromatic aminotransferase activity obtained from the Q-Sepharose column. See text and Fig. 1 for details.
[b] 1.8 M ammonium sulfate was used.

3. Results and discussion

3.1. Purification and molecular weight determination

When a cell-free extract of *P. furiosus* was fractionated on a Q-Sepharose column under anaerobic conditions, at least two major activity peaks were obtained when assayed for their ability to transfer the amino group from tyrosine to 2-ketoglutarate. As shown in Fig. 1, the second of these overlapped with the elution of aldehyde ferredoxin oxidoreductase, an enzyme we had previously purified from this organism [20]. The characterization of this second ArAT activity is currently under study and the results will be described elsewhere. The active fractions corresponding to the first peak of ArAT activity, which represented approx. one-third of the total ArAT activity, were combined and further purified. Since exposure to O_2 did not affect ArAT activity (data not shown), this and all subsequent manipulations were carried out under aerobic conditions. During the purification procedure, enzymatic assays were carried out in 200 mM potassium phosphate (pH 7.6), at 80°C, either using tyrosine (9.75 mM) or phenylalanine (19.5 mM) as substrates (Table 1).

The results of the purification procedure are given in Table 1. Note that, except for the crude extract, the values in Table 1 are only for the first peak of ArAT activity from the Q-Sepharose column. Thus, for the last four steps of the purification, the ratio of the two activities (using either phenylalanine or tyrosine as substrate) was constant (3.35 ± 0.25), and the final recoveries of activity and the purification factors were virtually identical for both activities (53.4% recovery and 41.3-fold purification for the phenylalanine-dependent activity and 55.1% and 42.6-fold for the tyrosine-dependent activity, where the Q-Sepharose fractions represents 100% activity and no purification). Thus, these results clearly demonstrate that it is the same enzyme that is catalyzing both activities throughout the final four purification steps. Based on the total aminotransferase activity in the crude extract (which contains a second ArAT), the purified ArAT represented 12% of the total phenylalanine aminotransferase activity and 7% of that dependent on tyrosine.

Following the last chromatographic step using S-Sepharose Fast Flow, the purified sample of ArAT*Pf* was subjected to gel filtration on a Superdex-200 column (Fig. 2) and was analyzed by SDS-PAGE (Fig. 3). These techniques revealed a single peak and a single band, respectively, indicating a high purity of the ArAT sample after just three chromatography steps. The apparent molecular weight of ArAT*Pf* estimated by gel filtration was 92000 ± 5000 (Fig. 2). The molecular weight of the subunit was 44000 ± 1000 as determined by SDS-PAGE (Fig. 3). These values are close to those measured using the same experimental techniques for TyrAT*Ec* (90000 and 46000) [1] but differ from those reported for rat liver TyrAT (160000 and 50000) [7,21]. A single unambiguous N-terminal amino acid sequence was obtained from purified ArAT*Pf* (L E S G P L N F E S Y S X E K A L T M K A X E V -), consistent with the presence of a single subunit. There was no homology between this sequence and the N-terminal sequences of the TyrATs from *E. coli* [3] and rat liver [22].

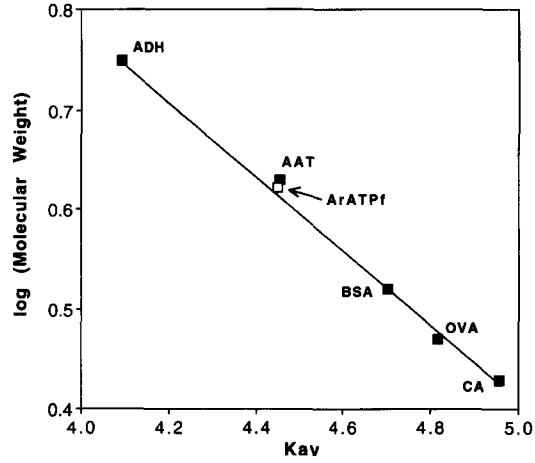

Fig. 2. Estimation of the molecular weight of purified *P. furiosus* aminotransferase. The column was Superdex 200 HiLoad (16/60) using 50 mM Tris-HCl (pH 8.0), containing 0.2 M NaCl as the buffer at a flow rate of 1 ml/min. The molecular weight standards were: ADH, alcohol dehydrogenase (150000); AAT, porcine cytoplasmic aspartate aminotransferase (92000); BSA, bovine serum albumin (66000), OVA, ovalbumin (45000); CA, carbonic anhydrase (29000).

Analysis and alignment of the amino acid sequences of TyrATEc and of rat liver TyrAT have shown that the bacterial enzyme is homologous to the central portion of the rat liver enzyme [22]. The higher molecular weight of rat liver TyrAT is accounted for by two extra peptides located at the amino and carboxy terminals of the polypeptide chain. Moreover, the extended conformation of the

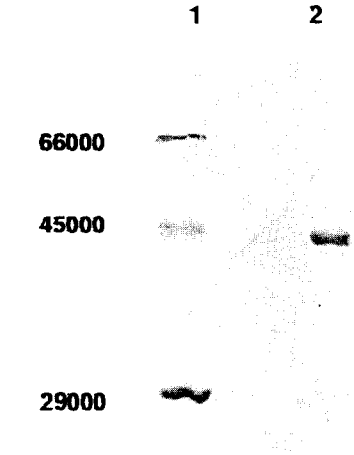

Fig. 3. Estimation of the molecular weight of the subunit of purified *P. furiosus* aminotransferase. Electrophoresis was carried out using an SDS-polyacrylamide gel (12.5% acrylamide). Lane 1: protein standards (0.5 μg total) containing bovine serum albumin, ovalbumin and carbonic anhydrase. Lane 2: 1 μg purified ArAT*Pf*.

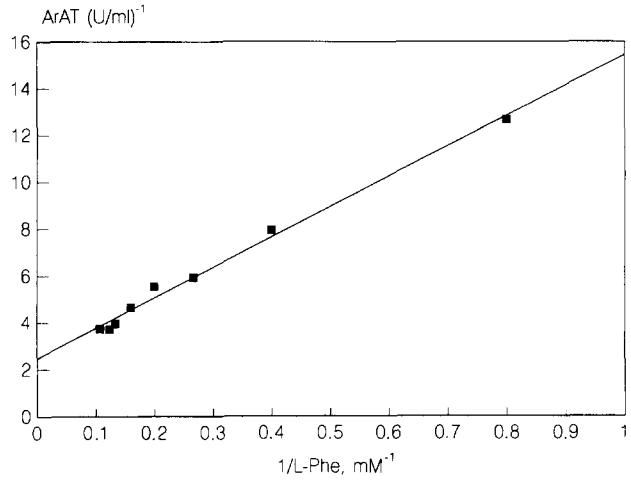

Fig. 4. Double reciprocal plot of phenylalanine aminotransferase activity. The activity of *P. furiosus* aromatic aminotransferase was determined as a function of phenylalanine concentration at 80°C varying the amount of phenylalanine and 2-ketoglutarate in the assay mixture and keeping the ratio between the concentrations constant ([phenylalanine]/[2-ketoglutarate] = 2).

extra peptides most likely affects the Stokes' radius of rat liver TyrAT such that its molecular weight is overestimated by gel filtration. Hence, rat liver ArAT, like the *E. coli* enzyme, is assumed to be a homodimer. The data described above for archaeal ArAT*Pf* therefore indicate that it structurally resembles bacterial TyrAT from *E. coli* and is devoid of the additional terminal peptides found in the eukaryotic TyrAT from liver.

3.2. Mechanism of reaction and substrate specificity

In order to investigate the mechanism of ArAT*Pf*, its activity was measured at 80°C in 0.2 M potassium phosphate (pH 7.6), containing 62.5 μM PM*P*, using phenylalanine and 2-ketoglutarate as substrates. Parallel primary plots were obtained when the concentration of one substrate was varied and the concentration of the other was kept constant, indicating the presence of a two step transfer mechanism for ArAT*Pf* (data not shown). This was confirmed by analogous measurements varying the concentration of both substrates at the same time but keeping their ratio constant. Under these conditions, the reciprocal of ArAT*Pf* activity depended linearly on the reciprocal of phenylalanine concentration (Fig. 4), as expected for a two step transfer mechanism.

Table 2 shows the K_m and k_{cat} values of ArAT*Pf* determined for phenylalanine, tryptophan, tyrosine and 2-ketoglutarate at 80°C. As in the case for the ArATs from *E. coli* [1] and *M. aeolicus* [4], ArAT*Pf* displays a broad specificity with aromatic amino acid substrates. In fact, the K_m values for the three substrates are very similar. For example, the ratio between k_{cat} and K_m calculated for phenylalanine is only 5-times higher than that calculated for tyrosine and 6-times higher than that for tryptophan.

Table 2
Kinetic parameters of *P. furiosus* aromatic aminotransferase

Substrate	K_m (mM)	k_{cat} (s^{-1})
Phenylalanine	1.15	253
Tryptophan	1.31	62
Tyrosine	2.1	72
2-Ketoglutarate	0.76	225

Activities were measured at 80°C in 200 mM potassium phosphate buffer (pH 7.6) containing 62.5 μM PMP and a saturating concentration of the nonlimiting substrate (9.4 mM for 2-ketoglutarate, and 19.5 mM for phenylalanine, tyrosine and tryptophan).

Therefore, ArAT*Pf* appears to be functionally more related to bacterial ArATs rather than to the rat liver enzyme as this is highly selective for one just one of the aromatic amino acids, tyrosine [7].

The activity of ArAT*Pf* was measured as a function of temperature (Fig. 5). Although the activity increases with increasing temperature in the range from 30°C to 95°C, the optimum was not determined and seems to be above 95°C. From the corresponding Arrhenius plot (Fig. 5), there is clearly a discontinuity in the slope centered at about 70°C. The calculated activation energies below and above the transition were 28 and 46 kJ/mol, respectively. Break points in Arrhenius-type plots have been observed at similar temperatures for other proteins from *P. furiosus*, including hydrogenase [12,23], ferredoxin [24] and glutamate dehydrogenase [25], although this was not the case with pyruvate ferredoxin oxidoreductase from this organism [15]. In the case of glutamate dehydrogenase, the transition was associated with a conformational change [25]. Analogous transition points are typically observed when analyzing enzymes from moderately thermophilic organisms [26,27]. Further analysis of ArAT*Pf* will be required to investigate the occurrence of a temperature induced conformational transition in this enzyme.

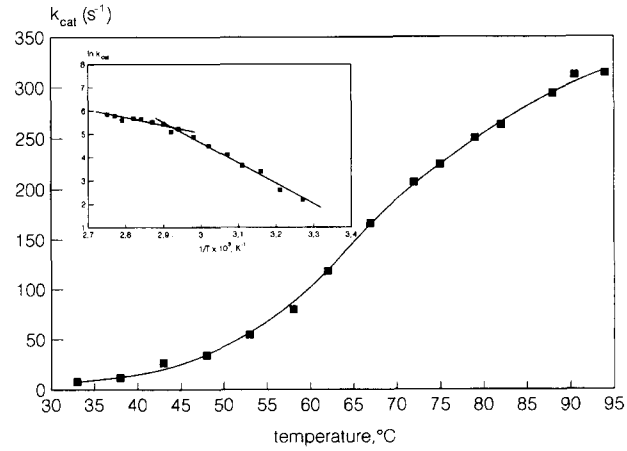

Fig. 5. Dependence of *P. furiosus* aromatic aminotransferase activity on temperature. The Arrhenius plot for the catalytic constants is given in the inset.

Table 3
Thermal stability of aromatic aminotransferase from *P. furiosus*

Sample	Relative activity [a] (%)			
	$T = 80°C$	$T = 95°C$		
	6 h	3 h	13 h	20 h
Holo (0.021 mg/ml) + PMP + 2-ketoglutarate [b]	100	100	73	34
Holo (0.021 mg/ml)	80	100	41	23
Apo (0.021 mg/ml) [c]	30	ND [b]	ND	ND

The purified enzyme (0.021 mg/ml in 50 mM Tris-HCl, pH 8.0) was incubated at 80 or 95°C for the indicated periods of time, using the holo or the apo form. Activity using phenylalanine as the substrate was measured at 80°C under standard conditions.
[a] Values refer to the activity of the untreated purified enzyme.
[b] The final concentrations were 0.1 mM for PMP and 2.0 mM for 2-ketoglutarate.
[c] The activity of the apoenzyme was determined after the reconstitution of the holoenzyme by incubating the sample for 1 h at 60°C in the presence of PMP and 2-ketoglutarate.
[b] ND, not determined.

3.3. Thermostability of ArATPf

Since *P. furiosus* grows at temperatures up to 105°C, close to the upper limit compatible with life, it was of some interest to investigate the thermal stability of ArAT*Pf*. Different experiments were therefore carried out to measure both the stability to reversible denaturation and the stability to irreversible denaturation caused by prolonged incubations at high temperatures. Reversible unfolding of ArAT*Pf* was studied by raising the temperature of an enzyme sample (0.54 mg/ml in 50 mM Tris-HCl, pH 8.0) from 25 to 95°C over a period of 20 min and measuring the ellipticity at 220 nm as a function of temperature. Independent experiments were carried out using the holoenzyme (in the pyridoxal form) and the apoenzyme. In both cases, the secondary structure of the protein remained essentially unchanged and the value of the ellipticity at 95°C was approx. 90% of that at 25°C. Technical problems prevented the measurement of the ellipticity above 95°C but it is likely that the melting temperature (T_m) for this enzyme is above 100°C.

Irreversible denaturation of ArAT*Pf* was followed by incubating the enzyme (0.021 mg/ml, 50 mM Tris-HCl, pH 8.0) at high temperatures in sealed vials and measuring the residual activity under standard conditions. As shown in Table 3, the holoenzyme (in its pyridoxal form) lost about 20% of its activity towards phenylalanine transamination after a 6 h incubation at 80°C. On the other hand, the apoenzyme was less stable under the same conditions as the residual activity (measured after reconstitution) was only 30% of the untreated control. However, the holoenzyme lost no activity when it was incubated for 6 h at 80°C in the presence of PM*P* and 2-ketoglutarate (0.1 and 2 mM, respectively). Thus, dissociation of PM*P* and/or lack of a substrate appears to have a destabilizing effect on the enzyme.

All of these data indicate that the holoenzyme and the apoenzyme maintain their secondary structures at temperatures as high as 95°C but at 80°C the apoenzyme more readily undergoes irreversible denaturation as a result of exposure of the PM*P*- and/or substrate-binding sites. Presumably this denaturation could be due to oxidations, isomerizations or hydrolysis of specific peptide bonds [28,29]. Further experiments carried out with ArAT*Pf* at 95°C confirmed the protective effect of PM*P* and 2-ketoglutarate and their relative effects. For example, ArAT*Pf* retained either 41% or 73% of its activity after 13 h at 95°C depending on the presence of a molar excess of PM*P* and 2-ketoglutarate, respectively. This experiment also served to compare the stability of ArAT*Pf* with that of AspAT from *S. solfataricus*, which shows a 50% loss of activity after 18 min at 95°C [30]. As expected, the thermal stabilities of the enzymes reflect the temperatures for optimal growth of these two archaea: 100°C for *P. furiosus* and 87°C for *S. solfataricus*. Hence, as might be expected, the enzyme from *P. furiosus* is therefore the most stable aminotransferase yet purified.

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