S-Adenosylhomocysteine Hydrolase from the Archaeon *Pyrococcus furiosus*: Biochemical Characterization and Analysis of Protein Structure by Comparative Molecular Modeling

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ABSTRACT **S-Adenosylhomocysteine hydrolase (AdoHcyHD) is an ubiquitous enzyme that catalyzes the breakdown of S-adenosylhomocysteine, a powerful inhibitor of most transmethylation reactions, to adenosine and L-homocysteine. AdoHcyHD from the hyperthermophilic archaeon *Pyrococcus furiosus* (PfAdoHcyHD) was cloned, expressed in *Escherichia coli*, and purified. The enzyme is thermoactive with an optimum temperature of 95°C, and thermostable retaining 100% residual activity after 1 h at 90°C and showing an apparent melting temperature of 98°C. The enzyme is a homotetramer of 190 kDa and contains four cysteine residues per subunit. Thiol groups are not involved in the catalytic process whereas disulfide bond(s) could be present since incubation with 0.8 M dithiothreitol reduces enzyme activity. Multiple sequence alignment of hyperthermophilic AdoHcyHD reveals the presence of two cysteine residues in the N-terminus of the enzyme conserved only in members of *Pyrococcus* species, and shows that hyperthermophilic AdoHcyHD lack eight C-terminal residues, thought to be important for structural and functional properties of the eukaryotic enzyme. The homology-modeled structure of PfAdoHcyHD shows that Trp220, Tyr181, Tyr184, and Leu185 of each subunit and Ile244 from a different subunit form a network of hydrophobic and aromatic interactions in the central channel formed at the subunits interface. These contacts partially replace the interactions of the C-terminal tail of the eukaryotic enzyme required for tetramer stability. Moreover, Cys221 and Lys245 substitute for Thr430 and Lys426, respectively, of the human enzyme in NAD-binding. Interestingly, all these residues are fairly well conserved in hyperthermophilic AdoHcyHDs but not in mesophilic ones, thus suggesting a common adaptation mechanism at high temperatures. Proteins 2005;58:815–825.**

Key words: S-Adenosylhomocysteine hydrolase; adenosylhomocysteine metabolism; hyperthermophilic enzymes; protein stability; disulfide bonds; NAD-binding domain; homology modeling

INTRODUCTION

S-Adenosylhomocysteine (AdoHcy) hydrolase (EC 3.3.1.1.) catalyzes the reversible breakdown of AdoHcy, a sulfur-containing nucleoside ubiquitously present in eukaryotes and prokaryotes, produced in all S-adenosylmethionine-dependent transmethylation reactions.[1–3] AdoHcy efficiently inhibits the enzymes involved in these reactions and therefore it plays an important role in regulating several biological processes that require S-adenosylmethionine (AdoMet).[1–3]

"Methylation ratio" refers to the relative levels of AdoMet and AdoHcy which are normally tightly regulated in the cells. The level of AdoMet is maintained at a concentration more that 10-fold higher than that of AdoHcy.[1–3] The maintainance of the correct "methylation ratio" is achieved by the conversion of AdoHcy to homocysteine and adenosine via the enzyme AdoHcy hydrolase (AdoHcyHD). Because this reaction is thermodynamically unfavorable,[1–3] the hydrolysis of AdoHcy depends on the efficient removal of both adenosine and homocysteine. In the past twenty years, intracellular AdoMet/AdoHcy ratio manipulation has been considered for use as an antiviral or antitumor therapeutical strategy.[4,5] As a result, drugs have been produced which exert their effect by causing AdoHcy to accumulate, primarily by inhibiting AdoHcyHD.[6,7] In addition to the antiviral effects, several AdoHcy hydrolase inhibitors have shown other effects of clinical importance including antiparasitic effects.[8,9]

In humans, homocysteine is produced solely from AdoHcy by AdoHcyHD and it has been reported that elevated plasma levels of homocysteine appear to be a risk factor for cardiovascular diseases.[10–11] Therefore, AdoHcyHD is of

Abbreviations: AdoHcy, S-adenosyl-L-homocysteine; AdoHcyHD, S-adenosylhomocysteine hydrolase; AdoMet, S-adenosylmethionine; SsAdoHcyHD, S-adenosylhomocysteine hydrolase from *Sulfolobus solfataricus*; PfAdoHcyHD, S-adenosylhomocysteine hydrolase from *Pyrococcus furiosus*.

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great interest as a target for the design of therapeutic inhibitors that may adjust the plasma homocysteine levels.

Very recently it has been reported the first case of human inherited deficiency of AdoHcyHD activity in a Croatan boy[12] where the effects of AdoHcy elevation are reported.

AdoHcyHD has been purified from many sources and complete coding sequences have been reported.[13,14] The enzyme is a highly conserved oligomeric protein with subunits of molecular masses ranging from 45 kDa to 55 kDa. Each subunit contains three domains and a tightly bound NAD cofactor which is involved in the catalytic mechanism.[15] Crystal structures of human[16,17] and rat[18,19] AdoHcyHD have been determined in both free and substrate-bound conformations, thus providing a detailed picture of conformational changes occurring during catalytic activity. Site-directed mutagenesis studies have been carried out to elucidate the structure and mechanism of action of recombinant rat liver AdoHcyHD.[20,21] Moreover, the three-dimensional structure of *Plasmodium falciparum* enzyme has been predicted by homology modeling.[22]

The analysis of the physiology, taxonomy and molecular biology of living organisms led to the classification of *Archaea* as a third evolutionary line together with the well known *Bacteria* and *Eucarya.*[23] Among the *Archaea*, hyperthermophiles are worthy of attention for their capacity for survival and reproduction at temperatures near 100°C. Therefore, these enzymes developed unique properties to maintain high thermostability and optimal activity at these temperatures.[24,25] These features make hyperthermophilic proteins useful models for understanding the molecular mechanisms of structural and functional adaptation of proteins to extreme temperatures and for designing stable proteins of biotechnological interest.[26]

Early evidence on AdoHcy metabolism in *Archaea* has been obtained in *Sulfolobus solfataricus*, an extreme thermoacidophilic archaeon.[27] In this microorganism AdoHcy hydrolase (SsAdoHcyHD) has been purified,[28] cloned,[29] and expressed in *E. coli.*[30] SsAdoHcyHD amino acid sequence lacks eight residues at the C-terminus[29] that are, instead, well conserved in all eukaryotic AdoHcyHD where they play important structural and functional roles.[14,16,18] Recently, it has been noted that thermophilic AdoHcyHD lack this region.[22]

To elucidate the mechanisms by which hyperthermophilic enzymes acquire their unusual thermostability and to increase our knowledge of the structure of AdoHcyHD, we have chosen as experimental model system AdoHcyHD from *Pyrococcus furiosus,* (PfAdoHcyHD) an anaerobic hyperthermophilic archaeon optimally growing at 100°C.[31]

This paper describes the expression in *E. coli* of the gene encoding AdoHcy hydrolase from *P. furiosus* and the purification and physicochemical characterization of the recombinant protein. Moreover, to investigate the possible involvement of cysteine residues of PfAdoHcyHD in disulfide bonds and to elucidate the functional significance of the C-terminal octapeptide, the three-dimensional structure of the enzyme has been constructed by homology modeling by using the crystal structure of human[16] and rat[18] AdoHcyHD as a templates.

MATERIALS AND METHODS

Bacterial Strains, Plasmid, Enzymes, and Chemicals

Plasmid pET-22b(+) and the NucleoSpin Plasmid kit for plasmid DNA preparation were obtained from Genenco. *E. coli* strain BL21(λDE3) was purchased from Novagen. *P. furiosus* chromosomal DNA was kindly provided by Dr. C. Bertoldo (Technical University Hamburg-Harburg, Germany). Specifically synthesized oligodeoxyribonucleotides were obtained from Primm. Restriction endonucleases and DNA-modifying enzymes were obtained from Takara. *Pfu* DNA polymerase was purchased from Stratagene. [8-^{14}C]-Adenosine (50 mCi/mmol) was supplied by the Radiochemical Centre (Amersham, U.K.). [8-^{14}C]AdoHcy was synthesized enzymatically[28] by labeled adenosine and non labeled L-homocysteine using partially purified rat liver AdoHcy hydrolase. Sephacryl S-300, AH-Sepharose 4B, AdoHcy, adenosine, L-homocysteine, O-bromoacetyl-N-hydroxysuccinimide and standard proteins used in molecular mass studies were obtained from Sigma. Cation exchanger cellulose phosphate P11 was purchased from Whatman. All reagents were of the purest commercial grade.

Enzyme Assay

The synthesis activity of AdoHcyHD was assayed by measuring the formation of [8-^{14}C]AdoHcy from [8-^{14}C]-adenosine in the presence of homocysteine, as previously described.[28] Unless otherwise stated, the standard incubation mixture contained the following: 10 μmol Tris-HCl, pH 7.4, 0.1 μmol EDTA, 11 nmol of [8-^{14}C]adenosine (5000 cpm/nmol), 400 nmol homocysteine, and the enzyme protein in a final volume of 100 μl. The incubation was performed in sealed glass vials for 5 min at 80°C, except where indicated otherwise. The vials were rapidly cooled in ice, and the reaction was stopped by the addition of 100 μl of 10% trichloroacetic acid. The mixture was then applied to a cellulose-phosphate column (0.6 × 2 cm) equilibrated in H_20. After washing with 10 mL 0.01 M HCl, which completely removed the unreacted adenosine, the [8-^{14}C]AdoHcy produced was eluted with 2.5 mL of 0.5 M HCl directly into scintillation vials and counted for radioactivity. Control experiments in the absence of the enzyme were performed in order to correct for adenosine hydrolysis. When the assays were carried out at temperatures above 80°C, the reaction mixture was preincubated for 2 min without the enzyme that was added immediately before starting the reaction. The hydrolytic activity of the enzyme was determined as reported previously by Porcelli et al.[28]

In all of the kinetic and purification studies, the amount of the protein was adjusted so that no more than 10% of the substrate was converted to product and the reaction rate was strictly linear as a function of time and protein concentration. One unit of enzyme activity was defined as the amount of enzyme which catalyzes the formation of 1-nmol AdoHcy/min at 80°C.

All enzyme reactions were performed in triplicate at 80°C. Michaelis-Menten type kinetics were observed for both the hydrolytic and synthetic reactions. Kinetic parameters (Km values) are obtained from at least 3-times experiments.

Cloning and Expression of the PfAdoHcyHD-Encoding Gene

The whole gene of AdoHcy hydrolase from *S. solfataricus* was used for BLAST search of the complete genome sequence of *P. furiosus* (http://combdna.umbi.umd.edu./bags.html) and a single gene was identified.

The coding region of PfAdoHcyHD was cloned into the pET-22b(+) expression vector via two engineered restriction sites (*Nde* I and *Bam* HI) introduced by PCR with the following primers 5′-GTGGTGAACCATATGGACTGTGG-3′, sense, and 5′-AAAATTATGGATCCAAGGTAATAAC-3′, antisense (the introduced restriction sites are underlined). Isolated genomic *P. furiosus* DNA (10 ng), hydrolyzed by *Bam* HI was used as a template. PCR amplification was performed with *P. furiosus* DNA polymerase and a Minicycler (Genenco) programmed for 29 cycles, each cycle consisting of denaturation at 95°C for 1 min, annealing at 45°C for 2 min and extension at 72°C for 2 min plus 5 sec/cycle, followed by an extension final step of 15 min at 72°C. The amplified gene (25 ng), hydrolyzed by *Nde* I and *Bam* HI was inserted into pET22b(+) (150 ng) cut with the same restriction enzymes. The recombinant plasmid was named pET-*PfAdoHcyhd*. The nucleotide sequence of the inserted gene was determined by MWG BIOTECH to ensure that no mutations were present in the gene. All DNA manipulations were carried out using the standard procedures and all enzymes were used under the conditions recommended.

For the expression of recombinant PfAdoHcyHD, an overnight culture of *E. coli* BL21 (λDE3) transformed with the plasmid pET-*PfAdoHcyhd* was used as 0.5% inoculum in 1 L Luria-Bertani medium[32] containing 100 μg/mL ampicillin at 37°C. At a late stage of cellular growth (when the culture reached an optical density of 3.0) isopropyl-β-D-thiogalactoside (IPTG) was added to 1-mM final concentration and the induction was prolonged for 16 h. Cells were harvested by centrifugation and lysed as described by Sambrook et al.[32] The cell debris was removed by centrifugation at 20,000 × g for 60 min at 4°C and the supernatant was used as a cell-free extract.

Preparation of AdoHcy-Sepharose

The preparation of AdoHcy-Sepharose was performed as described in Porcelli et al.[28] by treating AH-Sepharose 4B (5 g) with 1-mmol O-bromo-acetyl-N-hydroxysuccinimide and then coupling the resin with 100 mg of AdoHcy.

Purification of Recombinant PfAdoHcyHD

All the purification steps were performed at room temperature. Recombinant PfAdoHcyHD was purified in two steps. The cell-free extract of BL21 *E. coli* cells expressing PfAdoHcyHD was heated at 80°C for 10 min and centrifuged at 15,000 × g for 60 min. After dialysis overnight against 100 vol of 10 mM Tris/HCl (pH 7.4), the enzyme was applied to an affinity column of AdoHcy Sepharose (2 × 12 cm) prepared as described by Porcelli et al.[28] equilibrated with 20 mM Tris/HCl (pH 7.4). The column was washed stepwise with 50 mL of the equilibration buffer and then with the same buffer containing 0.5 M NaCl until the absorbance at 280 nm reached the baseline. AdoHcy hydrolase activity was then eluted with 20 mM Tris/HCl (pH 7.4) containing 0.5 M NaCl and 3 mM AdoHcy. Active fractions were pooled, concentrated and dialyzed extensively against 1000 vol of 10 mM Tris/HCl (pH 7.4).

Protein Analysis

Proteins were assayed by the Bradford method[33] using bovine serum albumin as standard. Protein eluting from the columns during purification was monitored as absorbance at 280 nm. The concentration of purified PfAdoHcyHD was estimated spectrophotometrically using $\epsilon_{280} = 57{,}750\ M^{-1}\ cm^{-1}$.

The molecular weight of the native protein was determined by gel filtration and nondenaturating polyacrylamide gel electrophoresis. Gel filtration was performed on a calibrated Sephacryl S-300 column (2.2 × 95 cm) equilibrated with 10 mM Tris-HCl (pH 7.4) containing 0.3 M NaCl at a flow rate of 4 mL/h. The column was calibrated by using standard proteins of known molecular weight. Nondenaturating polyacrylamide gel electrophoresis was carried out at pH 7.5 as reported by Cacciapuoti et al.[34] The gels were either stained with Coomassie Blue or cut into thin slices that were assayed for AdoHcy hydrolase activity by incubating in the assay mixture at 80°C for 10 min. The subunit molecular mass was determined by SDS polyacrylamide gel electrophoresis as described by Weber et al.[35] by using 12 or 15% acrylamide resolving gel and 5% acrylamide stacking gel. Samples were heated at 100°C for 5 min in 2% SDS, 5% 2-mercaptoethanol and run in comparison with molecular weight standards. Approximately 50 μg of purified protein, separated under denaturating conditions on a 15% SDS-PAGE, was electroblotted onto a PVDF membrane utilizing a Mini trans-blot transfer cell (Bio Rad) apparatus, stained with Coomassie brillant blue R-250 (0.1% in 50% methanol) for 5 min and destained in 50% methanol and 10% acetic acid for 10 min at room temperature. Stained protein bands were excised from the blot and their NH_2-terminal sequences were determined by automated Edman degradation on a pulsed liquid sequencer (model 473A, Applied Biosystem) connected in line to an HPLC apparatus for phenylthiohydantoin-derivative identification, following the procedures suggested by the manufacturer. The repetitive yield, based on stable amino acids, was approximately 95%.

Thermostability Studies

Enzyme thermostability was tested by incubating the protein in sealed glass vials at temperatures between 90°C and 100°C. Samples (2 μg) were taken at time intervals and residual activity was determined by the standard assay at 80°C. Activity values are expressed as a percentage of the zero-time control (100%).

Multiple Sequence Alignment and Homology Modeling

Protein similarity searches were performed using the data from Swiss-Prot and Protein Identification Resource (PIR) data banks. The multiple alignment was constructed using the Clustal method.[36]

Model building of PfAdoHcyHD monomer was performed with the SWISS-MODEL server,[37] using the structure of human[16] and rat[18] AdoHcyHD (1a7a and 1b3r) as templates. Tetrameric assemblage was obtained manually by superimposition of the modeled monomer with each subunit of the tetrameric template, using the program O[38] on a Silicon Graphics workstation. Side chains in close contacts were removed and manually rebuilt. The final model was energy-minimized by means of the GROMACS forcefield[39] to relieve steric clashes. The structure was validated with PROCHECK.[40] Docking of the NAD^+ cofactor into the structure of PfAdoHcyHD was performed superimposing the NAD^+-binding site of the rat enzyme to that of the model. Cartoons were generated using MOLSCRIPT.[41]

RESULTS AND DISCUSSION

Analysis of the Gene and Primary Sequence Comparisons

From the complete genome sequence of *P. furiosus* the gene *PF0343* encoding PfAdoHcy hydrolase was identified as a 1266-bp fragment that, when translated, encodes a protein of 421 residues with a predicted molecular mass of 47382 Da.

Homology between the AdoHcy hydrolase primary sequences from different organisms indicates that this enzyme is well conserved in evolution.

When the deduced primary structure of *P. furiosus* AdoHcyHD was compared with the enzymes present in GenBank data bases the highest identity was found with putative AdoHcy hydrolases from the hyperthermophilic *Archaea Pyrococcus horikoshii* (94%) and *Pyrococcus abyssi* (91%). A high identity (61%) was also found with AdoHcyHD from *Sulfolobus solfataricus*.

Among the related proteins isolated from various sources, PfAdoHcyHD shows high sequence homology with AdoHcyHD from *Homo sapiens* (46% identity), R*attus norvegicus* (46%) and *Rhodobacter capsulatus* (41%), a lower identity with *Plasmodium falciparum* (37%) and no significant identity with AdoHcy nucleosidase from *E. coli*.

As deduced from the gene, PfAdoHcyHD contains four cysteine residues per subunit. This feature is unexpected since cysteine is particularly sensitive to oxidation at high temperatures and is thought to contribute to the thermal destabilization of proteins.[42] In this respect, it is interesting to note that AdoHcyHD from the thermophilic archaeon *S. solfataricus* does not contain cysteine residues.[29] On the other hand, the availability of many completely sequenced hyperthermophilic genomes has indicated that cysteine residues are present in remarkable amounts in hyperthermophilic proteins. In these proteins, these thermolabile residues are probably protected against thermal inactivation by their burial in the protein interior or by their involvement in specific stabilizing interactions such as metal liganding or disulfide bridges.[25]

Figure 1 shows the multiple sequence alignment of PfAdoHcyHD with the enzyme from *Homo sapiens* and from 10 hyperthermophilic microorganisms. Among these, eight belong to the *Archaea* domain and two, namely *Aquifex aeolicus* and *Thermotoga maritima,* to the *Bacteria* domain.

On the basis of crystallographic data of the three-dimensional structure of human[16] and rat[18] AdoHcyHD the amino acid residues involved in the active site and in the NAD-binding site have been identified. The sequence alignment indicates that all residues that have been proposed to be part of the catalytic site are conserved in PfAdoHcyHD with the exception of Thr57 and Glu59 of *Homo sapiens* enzyme that are substituted by Glu55 and Lys57 respectively, in PfAdoHcyHD.

From a comparison of AdoHcyHD sequences, it appears that, in contrast to the human enzyme, all hyperthermophilic AdoHcyHD lack eight amino acid residues in the COOH-terminal region. This area, which is well conserved in all eukaryotic AdoHcy hydrolases, has been thought to play a role in the binding of the NAD cofactor. In fact, as reported for rat[18] and human[16] enzyme, this region forms a lid for the NAD-binding site, with Lys425/426 and Tyr429/430 respectively, making hydrogen bonds with the cofactor. Therefore, clear-cut differences exist between hyperthermophilic and mammalian AdoHcyHD whose significance must still be elucidated.

In spite of the high degree of identity among hyperthermophilic AdoHcyHD, the four cysteine residues of PfAdoHcyHD are conserved only in the enzyme from *P. horikoshii* and *P. abyssi*. Two of them are located in the NH_2-terminal region at position 3 and 8, while Cys221 and Cys278 are located into the coenzyme-binding domain where they substitute Val224 and Ile281 of human enzyme.

Cloning, Expression, and Purification of Recombinant PfAdoHcyHD

The PCR-amplified fragment of PfAdoHcy hydrolase was cloned into pET-22b(+). The recombinant PfAdoHcyHD was expressed in soluble form in *E. coli* BL21(λDE3) cells containing the pET-*PfAdoHcyhd* plasmid at 37°C in the presence of IPTG. The most favorable conditions for the expression of the enzyme were found to be when IPTG was added at the late stage of cellular growth and when the induction was prolonged for 16 h. Therefore, these conditions were chosen for the large scale production of recombinant PfAdoHcyHD and about 10 g of wet cell paste was obtained from 1 L liter of culture.

The recombinant PfAdoHcyHD was easily purified to homogeneity 48-fold by two-step purification procedure. The result of the large-scale purification is shown in Table 1. The first step in the purification of the protein from crude cell lysate was an optimized heat precipitation, made possible by the thermostability of the enzyme. As shown in Figure 2, which reports the analysis by SDS-polyacrylamide gel electrophoresis of recombinant

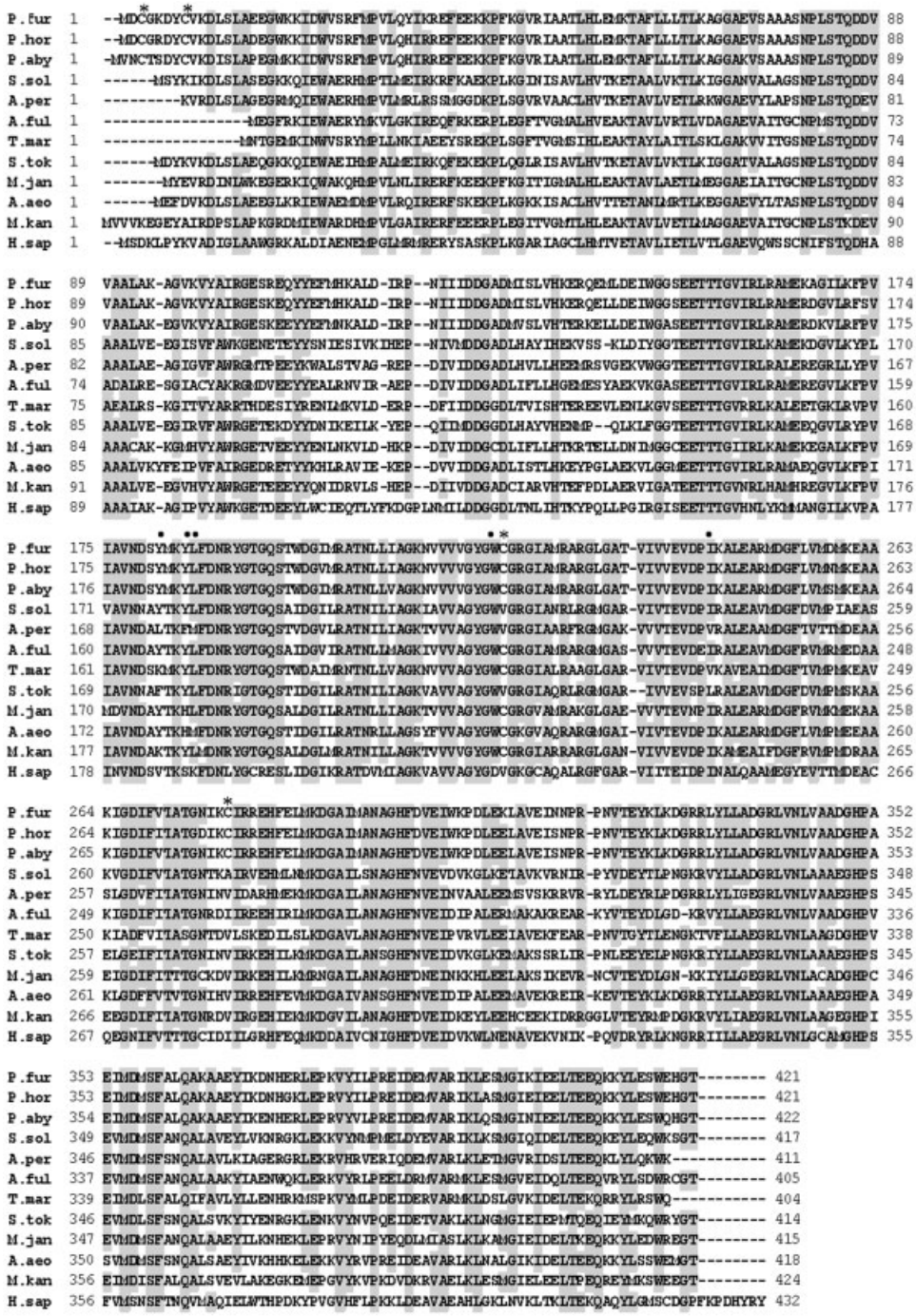

Fig. 1. Multiple sequence alignment of hyperthermophilic AdoHcyHD from different sources. Sequences: P.fur, *Pyrococcus furiosus*; P.hor, *Pyrococcus horikoshii*; P.aby, *Pyrococcus abyssi*; S.sol, *Sulfolobus solfataricus*; A.per, *Aeropyrum pernix;* A.ful, *Archaeoglobulus fulgidus*; T.mar, *Thermotoga maritima*; S.tok, *Sulfolobus tokoday*; M.jan, *Methanococcus jannaschi*; A.aeo, *Aquifex aeolicus*; M.kan, *Methanococcus kandleri*; H.sap, *Homo sapiens*. Numbers are the coordinates of each protein. Invariant and highly conserved positions are highlighted. Asterisks indicate the cysteine residues. Circles indicate the amino acid residues discussed in the text.

PfAdoHcyHD at different stages of purification, most *E. coli* thermolabile proteins can be denaturated and precipitated by heating and only minor contaminants of thermostable recombinant PfAdoHcyHD are detectable. The remaining impurities were removed by an affinity chromatography on AdoHcy-Sepharose. About 1 mg of enzyme preparation with a 19% yield was easily obtained from 1 L of culture.

TABLE I. Purification of Recombinant S-Adenosylhomocysteine Hydrolase from *P. furiosus*[†]

	Total protein (mg)	Total activity (units)	Specific activity[a] (units/mg)	Yield (%)	Purification (n-fold)
Crude extract	250	175	0.7	100	1
Heat treatment	27.6	135.1	4.9	77.2	7
AdoHcy-Sepharose	1.0	33.6	33.6	19.2	48

[†]A typical purification from 10 g of wet cells is shown.
[a]Specific activity is expressed as nmoles of AdoHcy formed per min per mg of protein at 80°C.

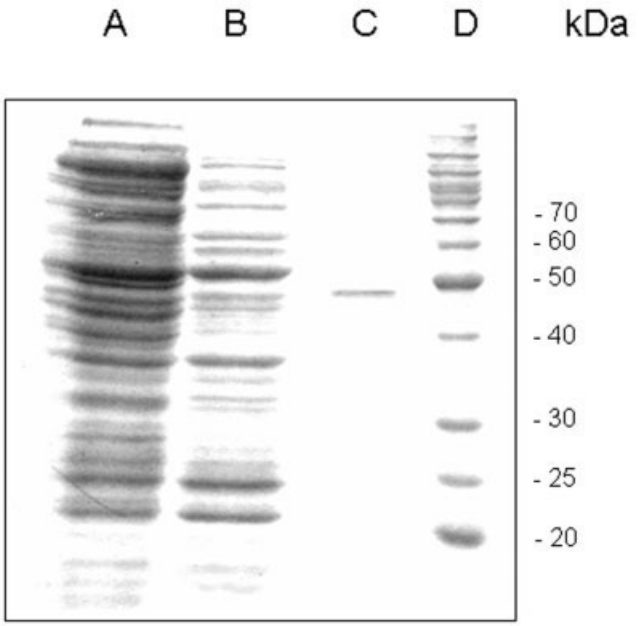

Fig. 2. SDS-polyacrylamide gel electrophoresis of recombinant PfAdoHcyHD at different stages of purification. Lane A, *E. coli* BL21 transformed with pET-AdoHcyHD, crude extract (20 μg); lane B, the same sample as lane A heated at 80°C for 10 min and cleared by centrifugation at 15,000g (10 μg); lane C, the same sample as lane B after affinity chromatography (2 μg) Lane D, molecular mass markers.

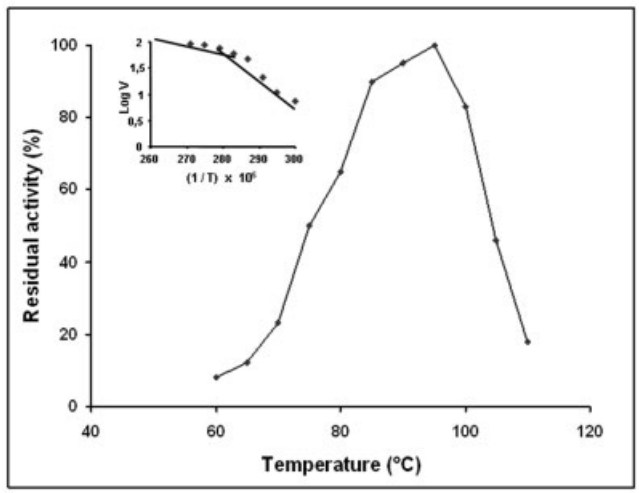

Fig. 3. The effect of temperature on PfAdoHcyHD activity. The activity observed at 95°C is expressed as 100%. The assay was performed as indicated under Materials and Methods. Arrhenius plot is reported in the inset. T is measured in Kelvin.

Biochemical Characterization

Several criteria were evaluated to assume the homogeneity of the enzyme. Under denaturating and native conditions the purified enzyme migrates as a single band on polyacrylamide gel electrophoresis. When assays are carried out on the material eluted from gel slices under native conditions the activity corresponds to the protein band. When purified enzyme is analyzed by isoelectric focusing on polyacrylamide gel (pH ranges 3–10 and 4.0–6.5), a single band is obtained at pH 5.1. The effect of pH on enzymatic activity was investigated in the pH range 5–10, with the optimum pH of the reaction being found at 7.4.

The apparent monomer mass of PfAdoHcyHD, as determined by SDS-PAGE, is 47 ± 1 kDa, which is in close agreement with that predicted from the gene. Gel filtration on an analytical column of Sephacryl S-300 suggests that, under native conditions, PfAdoHcyHD forms a tetrameric structure with a molecular mass of 190 ± 7 kDa. The molecular mass of PfAdoHcyHD is similar to that of mammalian AdoHcy hydrolase,[13,14,16,18] but differs from that of *Alcaligenes faecalis*, plants and parasites.[9,13,22] The N-terminal residue appears to be blocked, since repeated cycles of automated Edman degradation produces no detectable phenylthiohydantoin-derivatives.

The variation of initial velocity as a function of substrate concentration showed typical Michaelis–Menten kinetics for all substrates. Km values for adenosine (Km_{app} 4.8 μM), homocysteine (Km_{app} 140 μM) and AdoHcy (Km_{app} 14 μM), are comparable to those found for the enzyme from other sources.[1,2,4,5,28]

Thermoactivity and Thermostability

The temperature dependence of PfAdoHcyHD activity and its optimum temperature were determined by carrying out enzymatic assays at differing temperatures from 60°C to 120°C (Fig. 3). The enzyme is thermoactive with an optimum temperature of 95°C which is 5°C lower than the organism's optimum growth temperature[31] but still falls within the physiological range.

Two different activation energy-dependent processes occurred, below and above 77°C, as suggested by the biphasic Arrhenius plot (see inset in Fig. 3). The protein is thought to adjust itself to changes in thermal environment, generating enzymatic forms with different catalytic properties. This hypothesis has been verified by spectroscopic methods for propylamine transferase from *S. solfataricus*[43] and for poly(ADP-ribose) polymerase-like enzyme from *S. solfataricus*.[44] Further analysis of

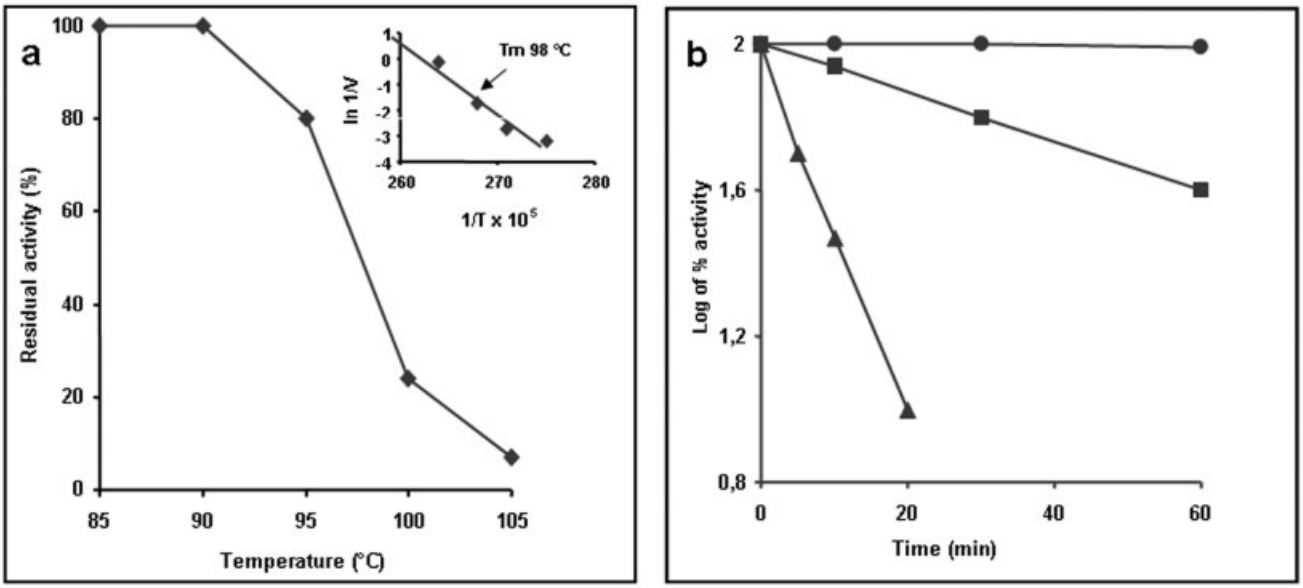

Fig. 4. Thermostability of PfAdoHcyHD. **a:** Residual PfAdoHcyHD activity after 10 min of incubation at temperatures shown. Apparent Tm are reported in the inset. **b:** Kinetics of thermal inactivation of PfAdoHcyHD as a function of incubation time. The enzyme was incubated at 90°C (-●-), 95°C (-■-), and 100°C (-▲-), for the times indicated. Aliquots were then withdrawn and assayed for the activity as described under Materials and Methods.

PfAdoHcyHD will be required to investigate the occurrence of a temperature-induced conformational transition in this enzyme.

The PfAdoHcyHD thermostability appears high even though lower than that of other enzymes characterized from *P. furiosus*.[45]

The PfAdoHcyHD stability to reversible denaturation was investigated by carrying out short-time kinetics of thermal denaturation. The diagram of the residual activity after 10 min of preincubation as a function of temperature, reported in Figure 4(a), is characterized by a sharp transition. From the corresponding plot (see inset), it is possible to calculate a transition temperature (apparent Tm) of 98°C. To study the thermostability properties in terms of resistance to irreversible thermal inactivation, the enzyme was incubated at a defined temperature from 90°C to 100°C. As reported in Figure 4(b), the enzyme decay obeys first order kinetic and shows half-lives of 50 min and 5 min when incubated at 95°C and 100°C, respectively. PfAdoHcyHD displayed a strong long-term heat resistance at 90°C retaining about 100% activity after 1 h incubation at this temperature.

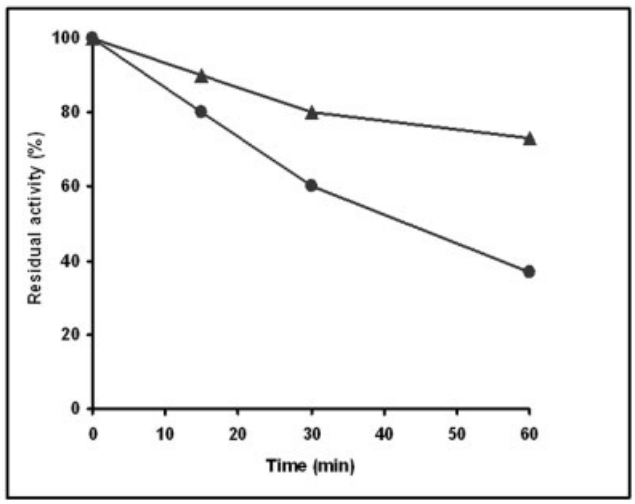

Fig. 5. Effect of reducing agents on PfAdoHcyHD. Thermostability of PfAdoHcyHD in dithiothreitol. The enzyme (2 μg) was incubated for different times in 20 mM Tris/HCl, pH 7.4, at 80°C in presence of 0.4 M dithiothreitol (-▲-), or 0.8 M dithiothreitol (-●-). Aliquots were withdrawn and assayed for the activity as described under Materials and Methods.

Reducing Agents and Disulfide Bonds

The catalytic process of PfAdoHcyHD does not involve thiol groups, since enzymatic activity is not affected by alkylating, mercaptide-forming or oxidizing thiol reagents even at relatively high concentrations (10 mM). Eukaryotic AdoHcyHD, instead, requires thiol reducing agents and is specifically and rapidly inactivated by thiol-blocking compounds.[1,2] Likewise, there are reports that one cysteine residue is involved in the catalytic mechanism of AdoHcyHD from rat liver where it modulates the oxidation state of the bound NAD.[46]

It is possible that in PfAdoHcyHD cysteine residues are involved in disulfide bridges. To verify this hypothesis, the thermal stability of purified PfAdoHcyHD was investigated by heating in the presence of reducing agents. At 50°C the enzyme is fully stable even at high concentrations of dithiothreitol (0.8 M) whereas it becomes susceptible to the effect of the reducing agents as the temperature rises. As reported in Figure 5, after 1 h of preincubation at 80°C a remarkable loss of activity (63%) is observed in the presence of 0.8 M dithiothreitol. These results and the observation that the growth temperature of *P. furiosus*

(100°C) is not compatible with the structural integrity of cysteines[42] prompted us to postulate that these residues could be linked by disulfide bonds. It has to be noted that these covalent links are not involved in the catalytic process since reducing agents do not affect PfAdoHcyHD activity even at high concentrations.

Although the presence of disulfide bonds has not been considered, until now, as a general feature of naturally occurring extremely thermophilic proteins, it has been reported as an important structural mechanism for the thermostability of MTAP from *S. solfataricus.*[47]

Homology Modeling

To investigate whether cysteine residues of PfAdoHcyHD could be involved in disulfide bonds and to study how the C-terminal octapeptide absence does influence the structural properties of the enzyme, we built a model of PfAdoHcyHD by using the human and rat enzyme crystal structures as templates.

Since the crystal structure of human and rat AdoHcyHD does not contain the first four residues at the N-terminus of each chain, this portion has not been determined in the model of PfAdoHcyHD.

As shown in Figure 6(a), the C-terminal regions of the four subunits of eukaryotic AdoHcyHD are positioned into the channel at the centre of the tetrameric structure, partially closing its entrance and crowding its interior.

The PfAdoHcyHD tetrameric model analysis reveals some features that differ significantly from those of mesophilic enzymes. Because of the lack of the C-terminal tail, the channel existing at the center of the tetramer is more empty, as shown in Figure 6(b). Since the absence of the C-terminal octapeptide is a common feature of hyperthermophilic AdoHcyHD, it seems reasonable that also in these enzymes, such a channel is characterized by a major void volume with respect to the mesophilic ones. It can be speculated that this structural feature could be useful for the hinge bending motion of the catalytic domain at high temperature, playing a role in the adaptation of AdoHcyHD at high temperatures. In fact, as proposed by Pearl and coworkers for the β-glycosidase from *Sulfolobus solfataricus,*[48] the solvent molecules within the channel formed at the subunits interface can absorb the energy of molecular collisions at high temperature and can dump the molecular vibrations, thus providing resistance to denaturation.

More subtle structural differences occur at the interfaces of the tetramer. Interestingly, Trp220 of PfAdoHcyHD is positioned to interact favorably with Tyr181, Tyr184, and Leu185 of the same subunit and with Ile244 from the adjacent subunit, forming a network of aromatic and hydrophobic contacts [Fig. 6(d)]. Furthermore, the side chains of these residues are bulkier than those occurring in the same positions of mesophilic AdoHcyHD, thus partly compensating for the effect of the C-terminal octapeptide deletion.

Remarkably, homology modeling analysis reveals the possible molecular strategy adopted by PfAdoHcyHD to substitute the interactions of the C-terminal tail involved in the binding and stabilization of NAD. In fact, Lys245 of PfAdoHcyHD could play the same role of Lys425/426 of the eukaryotic enzyme making two hydrogen bonds with the ribose in the adenosyl moiety of NAD and maintaining a positive electrostatic potential at that location. Similarly, Cys221, which is positioned in the pocket of NAD, could make two hydrogen bonds with O1 and O2 atoms of the phosphate of the NAD^+, replacing Val223/224 and Tyr429/430 respectively those of the rat and human enzyme. It is interesting to note, in this respect, that the involvement of the thiol group of Cys221 in the binding with the cofactor stabilizes this termolable residue to oxidation at the high growth temperature of the microorganism.

The model also shows that Cys278 occurs in the NAD-domain, and it is possible to speculate that its thiol group could to form a hydrogen bond with Thr272. Thus, the thiol group of both Cys221 and Cys278 seem stabilized by such interactions.

The remaining two cysteine residues, Cys3 and Cys8, which are conserved only in PfAdoHcyHD from *Pyrococcus* species, could form a disulfide bond. Since it has been demonstrated that the N-terminal region of mesophilic proteins is usually highly disordered and is thought to be the first portion of the protein that undergoes denaturation at high temperature,[49] the presence of a disulfide bond might increase its stability. This observation, together with the reported results on the thermoinactivation of PfAdoHcyHD in the presence of reducing agents, suggests that in AdoHcyHD from *P. furiosus,* as well as in the homologous AdoHcyHD from *pyrococcus* species, the formation of a disulfide bond in the N-terminal region could represent, in addition to other stabilizing interactions, a factor of thermostabilization.

CONCLUSIONS

In this paper, we present the cloning of the AdoHcyHD gene from *Pyrococcus furiosus*, the expression, purification, and biochemical characterization of the enzyme. The enzyme is a tetrameric protein with a molecular mass of 190 kDa and is composed by four apparently identical subunits. PfAdoHcyHD is a thermoactive and thermostable protein with catalytic features similar to those of eukaryotic AdoHcyHD. No loss of activity is observed in the presence of reducing agents indicating that thiol groups are not involved in the catalytic process. On the other hand, the enzyme thermostability is greatly reduced in the presence of dithiothreitol at 80°C suggesting the presence of disulfide bonds. The enzyme, in agreement with other hyperthermophiles AdoHcyHD, lacks eight amino acid residues in the COOH-terminal region.

The three-dimensional structure of PfAdoHcyHD is predicted by homology modeling procedure by using the X-ray crystal structure of rat and human enzymes as templates. The model suggests that a network of aromatic and hydrophobic contacts is present in the central channel formed at the interface among subunits. All hydrophobic and aromatic residues of the network and the residues potentially involved in the cofactor binding are fairly well conserved among the hyperthermophilic AdoHcyHD (Fig.1), but not in the mesophilic ones. These results

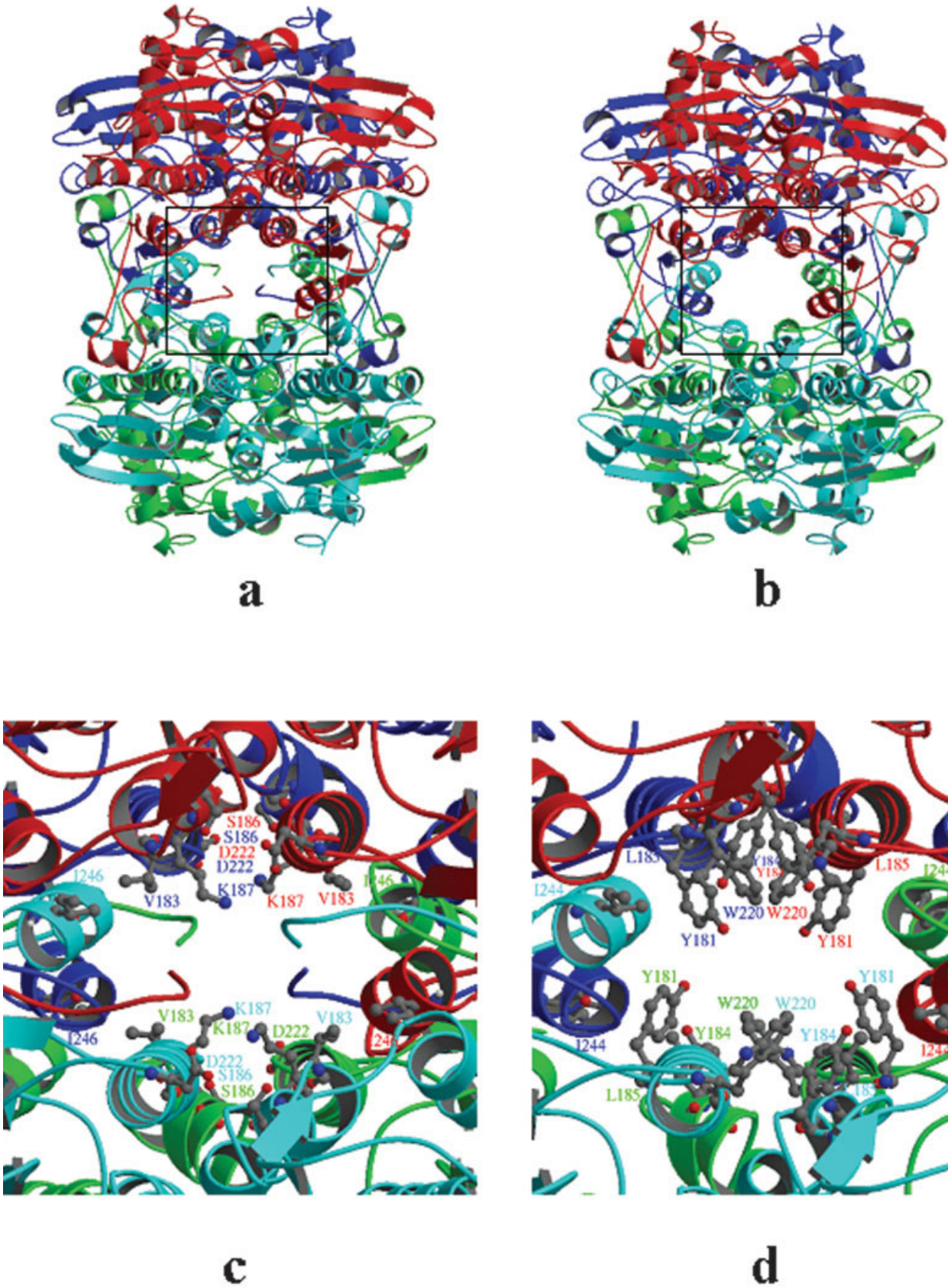

Fig. 6. Ribbon diagrams of the tetrameric structure of rat AdoHcyHD (**a**) and PfAdoHcyHD (**b**). The enlargements (**c**) and (**d**) show the individual amino acids involved in the network of hydrophobic and aromatic interactions in the central channel of rat AdoHcyHD and PfAdoHcyHD respectively. Subunits are indicated in orange, blue, green and cyan.

suggest a common adaptation mechanism of hyperthermophilic AdoHcyHD to be functionally active at high temperatures. Furthermore, among the four cysteines, Cys221 and Cys278 are stabilized in the binding pocket of NAD, while Cys3 and Cys8 in the N-terminal segment could form a disulfide bridge. The determination of the three-dimensional structure of PfAdoHcyHD could expand our understanding of this enzyme and confirm the hypotheses stated.

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