Multiple disulfide bridges modulate conformational stability and flexibility in hyperthermophilic archaean purine nucleoside phosphorylase

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5′-Deoxy-5′-methylthioadenosine phosphorylase from Sulfolobus solfataricus is a hexameric hyperthermophilic protein containing in each subunit two pairs of disulfide bridges, a CXC motif, and one free cysteine. The contribution of each disulfide bridge to the protein conformational stability and flexibility has been assessed by comparing the thermal unfolding and the limited proteolysis of the wild-type enzyme and its variants obtained by site-directed mutagenesis of the seven cysteine residues. All variants catalyzed efficiently MTA cleavage with specific activity similar to the wild-type enzyme. The elimination of all cysteine residues caused a substantial decrease of △H unfolding (850 kcal/mol) and T_m (39 °C) with respect to the wild-type indicating that all cysteine pairs and especially the CXC motif significantly contribute to the enzyme thermal stability. Disulfide bond Cys200–Cys262 and the CXC motif weakly affected protein flexibility while the elimination of the disulfide bond Cys138–Cys205 led to an increased protease susceptibility. Experimental evidence from limited proteolysis, differential scanning calorimetry, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing and nonreducing conditions also allowed to propose a stabilizing role for the free Cys164.

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1. Introduction

Hyperthermophilic organisms grow optimally at temperature near or above 100 °C. Therefore, their proteins must remain folded and functional at these high temperatures, keeping the required stability and structural flexibility. Understanding the mechanisms of this enhanced thermostability in such proteins has become a fundamental goal in the field of protein biochemistry. In fact, not only these proteins provided unique insights into the rules governing protein folding, stability and structural flexibility, but their peculiar structure–function properties are potentially significant for developing biotechnological applications through protein engineering. Studying different mechanisms by which proteins increase or decrease stability sheds light on the fundamentals of protein thermodynamics and can contribute to design new enzymes with desired stability [1–5]. The crystal structures of many hyperthermophilic proteins have been determined, and several factors responsible for their extreme thermostability have been proposed [6–10], including increases in the number of ion pairs and hydrogen bonds, core hydrophobicity and packing density, as well as the oligomerization of several subunits, and an entropic effect due to the relatively shorter surface loops and polypeptide chains. It has been recently reported that some proteins from hyperthermophiles are characterized by remarkably slow unfolding rates and it has been suggested that a kinetic barrier towards unfolding may be a common strategy used by many proteins to withstand extreme conditions [11,12]. However, although many theoretical and experimental studies have been carried out in the past, no general mechanism for increased thermostability was established to date. Proteins attain stability through a combination of many factors that contribute, in different proteins, to various extents.

In spite of the classical view that stabilizing disulfide bonds are rare in intracellular proteins from well known organisms due to the reducing chemical environment inside the cells [13], recent computational, structural and biochemical studies have pointed out the critical role of these covalent links in the structural stabilization of intracellular proteins of some hyperthermophilic Archaea and Bacteria [14–19]. The abundance of disulfides observed across hyperthermophilic organisms also stimulated research into the identification of the biochemical mechanisms related to disulfide maintenance. It has been demonstrated that specific
protein-disulfide oxidoreductases [20], structurally and functionally related to protein-disulfide isomerase (PDI) [21], play a key role in intracellular disulfide-shuffling in hyperthermophilic proteins.

Purine nucleoside phosphorylases (PNPs) are ubiquitous enzymes of purine metabolism that function in the purine salvage pathway of the cells [22,23]. All archaeal PNPs so far characterized, are extremely thermophilic and thermostable proteins with temperature optima and melting temperature above 100 °C and are stabilized by disulfide bridges [15,18,19,24].

5'-Deoxy-5'-methylthioadenosine phosphorylase II from the archaeon Sulfolobus solfataricus (SsMTAPII), a member of the archaeal family PNPs, is a hyperthermophilic enzyme that has been fully characterized by its physicochemical, kinetic and structural properties [24]. The presence in SsMTAPII of multiple intrasubunit disulfide bonds offers a good opportunity to elucidate the contribution of these covalent links in preserving the native conformation of a protein at extreme environmental temperature.

SsMTAPII is a homohexamer characterized by extremely high affinity towards 5'-deoxy-5'-methylthioadenosine (MTA), a sulfur-containing nucleoside generated from S-adenosylmethionine (AdoMet) mainly through polyamine biosynthesis [25]. SsMTAPII also accepts adenosine as substrate [24].

SsMTAPII is a tightly packed protein of 180 kDa, as shown by its tridimensional structure solved to 1.45 Å resolution (PDB entry code 2AVY) [26]. The hexameric structure of SsMTAPII is a dimer-of-trimers with one active site per monomer. The oligomeric assembly of the trimer and the monomer topology of SsMTAPII are almost identical to trimeric human 5'-deoxy-5'-methylthioadenosine phosphorylase [23]. Therefore, SsMTAPII can be regarded as the first reported hexamer in the trimeric class of PNP from Archaea. SsMTAPII contains seven cysteine residues per monomer. Four cysteine residues form two pairs of intrasubunit disulfide bridges, Cys138–Cys205 and Cys200–Cys262, near the C-terminus of the protein. The seventh cysteine, Cys164, is free. Each subunit contains in its C-terminal region a CXXC motif (i.e. a couple of cysteine residues separated by one amino acid X) as a typical feature. This motif is very close to the two pairs of disulfide bridges that are separated at a distance of 8.5 Å from the centers of disulfide bonds.

By enzymatic activity measurements it has been demonstrated that the substitution of these two cysteine residues (C259 and C261) with serine results in a significant decrease of thermal stability parameters of SsMTAPII [24] and that this unusual CXXC disulfide is necessary to obtain a complete unfolding reversibility of the protein [27]. The biochemical characterization of SsMTAPII has highlighted features of exceptional thermostability, with an optimum temperature for activity of 120 °C, an apparent melting temperature (Tm) of 112 °C and a notably high stability towards irreversible thermal inactivation processes. Moreover, SsMTAPII possess a remarkable resistance to sodium dodecyl sulfate (SDS), proteolytic cleavage, and guanidinium chloride (GdmCl)-induced unfolding [24].

In this study, the quantitative contribution of disulfide bonds to thermal stability of SsMTAPII was assessed by a comparative kinetic and calorimetric analysis of the wild-type enzyme and of its mutants obtained by site-directed mutagenesis of the seven cysteine residues in the monomer. Furthermore, using limited proteolysis we were able to probe the structural and dynamic differences among SsMTAPII and its cysteine-lacking mutants, therefore obtaining relevant information on the involvement of disulfide bridges in the enzyme conformational flexibility.

2. Experimental procedures

2.1. Bacterial strains, plasmid, enzymes and chemicals

Escherichia coli strain BL21 (DE3) was purchased from Novagen (Darmstadt, Germany). S. solfataricus chromosomal DNA was kindly provided by Prof. A. Gambacorta (Institute of Biomolecular Chemistry ICB-CNRCNR, Naples). Plasmid pET-22b (+) and the NucleoSpin Plasmid kit for plasmid DNA preparation were obtained from Genencor (Duren, Germany). Specifically synthesized oligodeoxyribonucleotides were obtained from MWG-Biotech (Ebersberg, Germany). Restriction endonucleases and DNA-modifying enzymes were obtained from Takara Bio, Inc. (Otsu, Shiga, Japan). Polyfu DNA polymerase was purchased from Stratagene (La Jolla, CA, USA).

MTA was prepared from AdoMet [28]. Nucleosides, purine bases and proteasome K were obtained from Sigma (St. Louis, MO, USA). Standard proteins were supplied by Applied Biological Materials Inc. (Richmond, BC, Canada). PIPES piperazine–N,N′-bis(2-ethanesulfonic acid) was obtained from Applichem (Darmstadt, Germany). 3-(1-Pyridinio)-1-bis(2-ethanesulfonic acid) was purchased from Sigma (St. Louis, MO, USA). Standard proteins were supplied by Applied Biological Materials Inc. (Richmond, BC, Canada).

2.2. Site-directed mutagenesis, expression and purification of SsMTAPII mutants

The sequence of SsMTAPII gene (GenBank™ accession number AE006641) from S. solfataricus was previously inserted in the pET-11a cloning site of the pET-22b (+) expression vector [24]. Site-directed mutants were prepared following the QuickChange site-directed mutagenesis protocol (Stratagene, La Jolla, CA) using the construct pET-SsMTAPII as a template. Primers for site-directed mutagenesis were designed according to the instructions for the Quick-Change kit. SsMTAPII mutants Cys262Ser (mut1) and Cys259Ser:Cys261Ser (mut2) were obtained by mutating Cys262 and Cys259-Cys261, respectively, in the wild-type SsMTAPII gene (GenBank™ accession number AE006641) from S. solfataricus. The mutants Cys259Ser:Cys261Ser:Cys262Ser (mut3), Cys259Ser:Cys261Ser:Cys262Ser:Cys200Ser (mut4), Cys259Ser:Cys261Ser:Cys262Ser:Cys200Ser (mut5) were obtained by mutating Cys259-Cys261 and Cys200-Cys262, respectively, in the wild-type SsMTAPII gene (GenBank™ accession number AE006641) from S. solfataricus. The mutants Cys259Ser:Cys261Ser:Cys262Ser:Cys200Ser (mut6), Cys138Ser-Cys164Ser (mut7), Cys138Ser-Cys164Ser (mut8) and Cys138Ser-Cys164Ser (mut9) were obtained by mutating Cys259-Cys261 and Cys200-Cys262, respectively, in the wild-type SsMTAPII gene (GenBank™ accession number AE006641) from S. solfataricus.

2.3. Enzyme preparation and assay

MTA phosphorylase activity of SsMTAPII and its mutants was determined by measuring the formation of adenine from MTA. Unless otherwise stated, the standard incubation mixture contained the following: 50 mM Hepes pH 7.4, 5 mM MTA, 5 μM NADP, 5 μM NADPH, 5 μM MgCl2, 10 μg/ml KPN and enzyme concentration, 1 cm 1 calculated at 280 nm for the hexameric protein [22].
the enzyme protein in a final volume of 200 μL. The incubation was performed in sealed glass vials for 5 min at 70 °C. The vials were rapidly cooled in ice, and the reaction was stopped by the addition of 50 μL of 3% (v/v) trifluoroacetic acid. Control experiments in the absence of the enzyme were performed in order to correct for spontaneous nucleoside hydrolysis. The formation of adenine base was monitored by measuring the percentage of the absorbance integrated peak area of adenine base formed with respect to the total (nucleoside + purine base) absorbance integrated peak areas. In all of the kinetic and purification studies the amounts of the protein was adjusted so that no more than 15% of the substrate was converted to product and the reaction rate was strictly linear as a function of time and protein concentration. All enzyme reactions were performed in triplicate. Values given are the average from at least three experiments with standard errors.

2.4. Temperature dependence and thermostability of activity

The temperature dependence of SsMTAPII and its mutants was studied by measuring the enzymatic activity in the temperature range from 40 °C to 120 °C. Enzyme thermostability was investigated by carrying out short time kinetics of thermal denaturation. The proteins were pre-incubated for 5 min in sealed glass vials at temperature between 50 °C and 120 °C in an oil bath. Residual activity was determined in standard conditions by the addition of aliquots from each preincubated sample.

2.5. Differential scanning calorimetry

Measurements were performed using a MicroCal VP-DSC instrument at a scan rate of 90 K h⁻¹ and under ~25 psi positive cell pressure. Samples were dialyzed overnight against 30 mM MOPS, pH 7.0. After dialysis, both the sample and the reference buffer were brought to 1.5 M NDSB, efficient to prevent protein aggregation, and to 0.5 M GdmCl at about 0.2–0.3 mg/mL final protein concentration. Thermograms normalized for protein concentration were analyzed for T max (top of the transition) and ΔH cal (area under the transition) using the MicroCal Origin software (version 7).

2.6. Dynamic light scattering

Dynamic light scattering data were recorded on a DynaPro NanoStar instrument (Wyatt Technology Corporation) operated in batch mode and fitted with a laser beam emitting at 658 nm with power autoattenuation. The scattering angle was 90° for the DLS avalanche photodiode. Samples were filtered on Whatman Anotop 10 inorganic membrane (0.02 μm cut off) and loaded into a 10 μl quartz microcuvette. Data were averaged from 20 acquisitions of the scattered light intensity during 5 s. Scattering data were analyzed using DYNAMICS v. 7.1.1.3 software (Wyatt Technology Corp.) that includes the DYNALS module for distribution analysis in photon correlation spectroscopy.

2.7. Circular dichroism spectroscopy

Circular dichroism (CD) spectra were recorded with a Jasco J-715 spectropolarimeter (Model PTC-348WI). The spectropolarimeter was calibrated with an aqueous solution of d-10-(+)-camphorsulfonic acid at 290 nm [33]. Molar ellipticity per mean residue, [θ] in deg cm²d·mol⁻¹, was calculated from the equation: [θ] = [θ]obs ·mrw ·(10·l·C)⁻¹, where [θ]obs is the ellipticity measured in degrees, mrw is the mean residue molecular weight (1116 Da), C is the protein concentration in g/mL and l is the optical path length of the cell in cm. A protein stock solution of each protein (0.5 mg/mL) in piperazine-N,N'-bis(ethanesulfonic acid) (PIPES buffer) 10 mM, pH 7.4, was diluted into buffer to achieve a normalized starting ellipticity of — 13.0, using a final protein concentration of 0.2 mg/mL. CD spectra in the far-UV region were recorded from 190 nm to 250 nm in a 0.1 cm path length cell with a final protein concentration of 0.2 mg/mL. Near-UV CD spectra were recorded from 250 nm to 320 nm using about 2 mg/mL protein samples and 0.5 cm path length cells. CD spectra were recorded with a time constant of 4 s, a 2 nm bandwidth and a scan rate of 20 nm/min; the signal was averaged over at least three scans and baseline was corrected by subtracting a buffer spectrum.

2.8. Limited proteolysis

Limited proteolysis experiments were carried out by incubating SsMTAPII and its mutants with proteinase K. Enzymatic digestion was performed in 10 mM Tris–HCl pH 7.4, at 37 °C. The final mass ratio of substrate protein to protease was 67:1. At different incubation times the hydrolysis was stopped by the addition of phenylmethysulfonyl fluoride (PMSF) (final concentration 250 μM) and the samples were assayed for MTA phosphorylase activity at 70 °C. The digested material (5 μg) was submitted to SDS-PAGE followed by staining with Coomassie brilliant blue R-250. For the amino acid sequence analysis, samples of the digested protein after SDS-PAGE, were electrophoretically blotted onto a polyvinylidene fluoride membrane utilizing a Bio-Rad Mini trans–blot transfer cell apparatus (Bio-Rad, Hercules, CA, USA). Transferred proteins were stained with Coomassie blue (0.1% in 50% methanol) for 5 min, destained in 50% methanol and 10% acetic acid solution for 30 min at room temperature and allowed to air dry. Stained protein bands were excised from the blot and their N-terminal sequences were determined.

3. Results and discussion

3.1. Design and general properties of SsMTAPII disulfide variants

Results from a detailed spectroscopic characterization of SsMTAPII and from thermal and GdmCl-induced unfolding/refolding experiments have demonstrated that disulfide bonds are crucial for the structural stability of the enzyme and that the denaturation of SsMTAPII is not a reversible equilibrium [34]. Nevertheless, both the chemical and thermal unfolding of the enzyme become reversible processes under reducing conditions, therefore suggesting that the loss of these covalent links weakens the tightly compact structure of the native protein and allows the reversible transition between the native and denatured states before modifications of protein covalent scaffold induced by high temperatures and chemical denaturants that cause the irreversible protein denaturation [34]. On the basis of these results it appears evident that, although the folding of a protein is a spontaneous in vivo process, the structural compactness of SsMTAPII and the presence of multiple disulfide bonds do not allow a reversible denaturation in vitro. This impairs the measurement of the parameters associated with protein thermodynamic stability that, instead, requires fully reversible protein unfolding.

In an attempt to make the heat-induced unfolding of SsMTAPII reversible, we have designed a variant in which all the seven cysteine residues of the enzyme are substituted by serine. The protocol for the synthesis of this mutant protein proceeded through the sequential insertion of single point mutations. This allowed to obtain all possible cysteine-lacking variants. Fig. 1 summarizes the cysteine/disulfide mapping of SsMTAPII variants. When compared to the wild-type, mut1 lacks one disulfide bond (C200–C262); mut2 lacks the CX motif (C259–C261); mut3 lacks both the disulfide bond C200–C262 and the CX motif; mut4 lacks two disulfide bonds (C200–C262 and C138–C205) and the CX motif, and displays a second free cysteine (C138) in addition to the native C164; mut5 shows only C164. Finally, mut6 represents a SsMTAPII variant lacking all cysteine residues. The synthesis of all these mutant proteins provided the opportunity to assess the effect of each substitution on the conformational stability and flexibility of
SsMTAPII using kinetic and calorimetric characterizations and limited proteolysis.

The mutant proteins were expressed in native form in *E. coli* BL-21 cells. Large scale production was performed as described for wild-type SsMTAPII [24]. All proteins were found to be homogeneous on analysis on SDS-PAGE and showed, under either native or denaturing conditions, Mr values identical to the wild-type. Moreover, all SsMTAPII variants catalyzed efficiently MTA cleavage with specific activity values similar to the wild-type enzyme (Table 1).

### 3.2. Spectroscopic characterization of SsMTAPII disulfide variants

To analyze the effect of mutations on protein structure, a structural characterization of all enzyme variants was performed by circular dichroism (CD). As reported in Fig. 2A, the far-UV CD spectrum (190–250 nm) of mut6, i.e. the cysteine-free variant, appears almost indistinguishable from that of the wild-type, showing a similar positive band at 195 nm and a broad negative band centered at 222 nm, indicative of the presence of both α- and β-secondary structure elements. The same features have been observed for all SsMTAPII mutants (data not shown) indicating that the backbone conformation of the mutant proteins is similar to the wild-type. Moreover, the almost complete overlap of the near-UV CD spectra of the wild-type and of mut6, recorded in PIPES buffer 10 mM pH 7.4 at 25 °C (Fig. 2B) indicates that these proteins are isomorphous in their native states and that the complete removal of the disulfide bridges caused no significant change in their three-dimensional structure. These results, together with previous investigations [24], demonstrate that none of the disulfide bonds are critically involved in the correct SsMTAPII folding into the catalytically active conformation.

### 3.3. Kinetic characterization of the thermal properties of SsMTAPII disulfide variants

In order to compare the thermal features of the wild-type and of its variants in terms of thermostability, the temperature dependence of SsMTAPII activity was recorded for all variants. Subsequently, the effect of amino acid substitutions on enzyme thermostability was analyzed by short time kinetics of thermal denaturation. The plot of the residual activity after 5 min of preincubation as a function of temperature allowed to calculate the temperature for half inactivation (50% residual activity). As reported in Table 1, the mutations significantly reduce the optimal temperature for the catalytic activity, which decreases from 120 °C (wild-type) to 107 °C (mut6). Moreover, the elimination of the two disulfide bridges and the CXC motif causes a strong destabilization for the folded structure of the enzyme, as inferred from the temperature for half inactivation, which decreases from 112 °C (wild-type) to 73 °C (mut6).

### 3.4. Role of disulfide bridges in thermal stability assessed by differential scanning calorimetry

To elucidate the stabilization mechanisms of hyperthermophilic proteins, the stability in solution should be quantitatively determined and the energetic aspects stabilizing the proteins from a thermodynamic point of view should be elucidated. The measurement of parameters associated with protein thermodynamic stability requires fully reversible protein unfolding. Thermal unfolding of hyperthermophilic proteins, however, is often followed by rapid irreversible denaturation [35–37] as a consequence of formation of insoluble aggregates due to the sticky,
hydrophobic nature of the exposed surfaces of the unfolded protein. Therefore, only few of such proteins, mainly small single-domain proteins, have been characterized in terms of their thermodynamic stability profiles [5].

Differential scanning calorimetry (DSC) allows a fine thermodynamic analysis of heat-induced unfolding of macromolecules and has significantly contributed to the elucidation of protein energetics [38]. Microcalorimetric studies of hyperthermophilic proteins are challenging as they unfold at the upper technical temperatures of DSC devices and are prone to massive aggregation at these high temperatures. In this study, we found suitable conditions to record reliable thermograms of SsMTAPII and of its mutants by using low protein concentrations (~0.2–0.3 mg/mL) and fast scan rate (90 °C/h), and addition of a non-detergent sulfobetaine (1.5 M) [39] and of GdmCl (0.5 M) to prevent aggregation. As shown in the thermograms reported in Fig. 3, these experimental conditions delay and temporarily prevent protein aggregation in the microcalorimetric cell, allowing the recording of a stable post-transition baseline and the analysis of the heat-absorption peak. Nevertheless, thermal unfolding of SsMTAPII and all of its variants was found to be irreversible (after cooling and re-scan of the sample) presumably caused by the presence of aggregates. The reversibility of the unfolding process has therefore limited the analysis to calorimetric parameters $T_{\text{max}}$ (temperature at the top of the transition) and $\Delta H_{\text{cal}}$ (area under the transition, corresponding to all enthalpy-driven interactions broken during unfolding), that are reported in Table 2. Attempts to perform DSC under reducing conditions were unsuccessful as a result of the large heat background produced by reducing agents.

SsMTAPII displays a strong thermal stability as inferred from both the high $T_{\text{max}}$ (111.6 °C) and calorimetric enthalpy (1110 kcal/mol) values. Disrupting the disulfide bond C200–C262 (leaving a free C200) in mut1 destabilizes the enzyme: $T_{\text{max}}$ decreases by 12 °C and $\Delta H_{\text{cal}}$ is reduced to about 66% of the wild-type value. However, removal in mut2 of the CXC motif C259–C261 has an even more drastic destabilizing effect, leaving only 35% of SsMTAPII $\Delta H_{\text{cal}}$ value (393 kcal/mol). As both cysteines are only separated by a serine in the sequence, such a stabilization of SsMTAPII via a structural disulfide bond appear unrealistic. This rather suggests a functional role of this unusual disulfide in protein stability and further confirms previous results [26,27,34] demonstrating that the CXC motif in SsMTAPII can form a third disulfide bond that mimic PDI [21] to rescue the possible damage to the other two disulfide bridges.

Removal of both the above mentioned disulfide bridge C200–C262 and the CXC motif in mut3 further decreases $T_{\text{max}}$ (85.5–90.2 °C) without significant alteration of $\Delta H_{\text{cal}}$ with respect to mut2 but mainly results in a biphasic unfolding transition, indicating the presence of two domains of distinct stability in this mutant enzyme.

Additional disruption of the disulfide bond C138–C205 (leaving a free C138) in mut4, decreases the stability of both domains observed in mut3 that now appear completely resolved. Surprisingly, removal of C138 in mut5 stabilizes this mutant with respect to mut4 by increasing both the $\Delta H_{\text{cal}}$ (374 kcal/mol) and $T_{\text{max}}$ (76.4–88.9 °C) values.

In the case of mutants mut4 and mut5, which display two well resolved transitions, the possibility that the first transition could correspond to the hexamer dissociation was addressed by dynamic light scattering in the same buffer (data not shown). However, only an increase of the hydrodynamic radius was recorded in the temperature range of these first transitions, without evidence for the appearance of...
monomers. It can be concluded that the first DSC transition for both mutants reflects an unfolding event.

The observation that the presence of the free cysteine C164 makes mut5 more stable than mut4 suggests two different hypotheses. The first is that in mut4 Cys138 probably performs unfavorable interactions with its immediate environment in the absence of its partner in the disulfide bond. The second is that in mut5 the absence of disulfide bridges may allow C164 to form new stabilizing disulfide bonds(s). Confirmation of the latter hypothesis has been provided by the results of SDS-PAGE of mut5 carried out under reducing and nonreducing conditions. As shown in Fig. 4, when the experiment was carried out in the absence of 2-mercaptoethanol, a protein band at about 75 kDa (lane 4) appears in addition to that at 30 kDa (lane 3) observed under reducing conditions. These data are consistent with the presence of structural blocks or units of distinct stability that unfold independently.

In conclusion, each disulfide bond contributes to varying degrees to the high Tm value of SsMTAPII. Moreover, the CXC motif also contributes to the enthalpic stabilization of SsMTAPII by about 65%, as inferred from the ΔHcal values of mutants lacking this motif.

### 3.5. Role of disulfide bridges in conformational flexibility assessed by limited proteolysis

The protein native state prevailing under physiological conditions is not a single molecular species but is better described by an ensemble of statistically populated conformers. In this context, “flexibility” refers to these fluctuating conformations of comparable energy that can be interconverted with a series of small changes. The larger the flexibility, the larger is the population of conformers and the lower is the energy barrier for interconversion between them [40].

On the basis of the three-dimensional structure of several proteins, it has been proposed that primary sites of proteolytic chain cleavage do not occur within chain segments having regular secondary structure (helices and sheets) but rather at the exposed loops [41]. A correlation between cleavage sites and sites of segmental mobility of the polypeptide chain has also been found [41]. Proteolytic enzymes, therefore, can be used to identify the sites of enhanced flexibility or of local unfolding propensity in the polypeptide chain [42].

We have previously demonstrated by limited proteolysis that SsMTAPII possesses only one proteolytic cleavage site localized in the C-terminal region and that the C-terminal peptide is necessary for the integrity of the active site [24]. It has been also demonstrated that the binding of MTA induces a conformational transition that protects the enzyme against protease inactivation [24]. It is interesting to note that the C-terminal region of SsMTAPII is highly flexible. In fact, as highlighted by the three-dimensional structure, the electron density of the C-terminus is relatively weak and the average B-factor of residues 262–270 is higher than the overall B-factor of the main chain [26].

To assess the native conformation of the SsMTAPII variants and to elucidate how disulfide bridges, CXC motif, and Cys164 affect the structural flexibility of the enzyme, each variant was subjected to limited proteolysis in comparison with the wild-type. The time course of proteolysis was investigated by measuring the residual activity after incubation with proteinase K at 37 °C, followed by SDS-PAGE of the digested material. As shown in Fig. 5A, the proteolytic pattern of the SsMTAPII variant lacking all cysteine residues (mut6) appears more complex than that of the wild-type. After 5 min incubation, the protease produced two major fragments among several proteolytic bands (lane 5). The first fragment, with a molecular mass slightly higher than that observed in wild-type (lane 2), probably marks the region where the unfolding process is initiated. The second fragment at about 18.5 kDa is rapidly

<table>
<thead>
<tr>
<th>Protein</th>
<th>ΔHcal (T1) kcal mol⁻¹</th>
<th>Tmax °C</th>
<th>T1max °C</th>
<th>T2max °C</th>
<th>ΔHcal (T2) kcal mol⁻¹</th>
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For biphasic thermograms, Tmax and ΔHcal (T1, T2) are given separately. Errors are ±10% on ΔHcal and ±0.2 °C on Tmax.
degraded as the proteolysis proceeds and is likely a product of the proteolytic cleavage of the first fragment since, when analyzed by Edman degradation, the N-terminus is preserved. This also indicates that in mut6, the proteolytic cleavage site is localized in the C-terminal region as for the wild-type. After 30 min incubation, the first fragment (lane 7) becomes abundant, and the residual activity drops to 23.6% (Fig. 5B). By analogy with the wild-type (lane 3, Fig. 5A), when the experiments were carried out in the presence of the substrate MTA, mut6 remained completely active and the proteolytic process did not occur (lane 8) indicating that, although the overall structure of mut6 is quite similar to that of the wild-type, the loss of the two disulfide bonds, the CXC motif, and Cys164 causes an obvious disturbance of the native state resulting in a higher protease susceptibility and in a different proteolytic pattern.

It was also found that the relative contribution of each disulfide bond to enzyme flexibility depends on its location within the polypeptide chain. After 30 min of incubation with protease K, mut1 and mut2 (lanes 2 and 4 in Fig. 5C) showed a primary cleavage site and a proteolytic pattern similar to that of the wild-type (lane 2 in Fig. 5A) with comparable values of residual catalytic activity (Fig. 5B). These data indicate that the disulfide bond Cys200–Cys262 and the CXC motif exert only a moderate effect on the susceptibility of SsMTAPII to the proteolytic attack, in spite of the significant role played by these covalent links on enzyme thermostability (Table 2). It should be pointed out that Cys262 is positioned in a already very flexible region of the enzyme [26]. Similarly, the loop including Cys259 and Cys261 is located in a highly disordered area whose flexibility is crucial to allow the CXC motif to position very close to the two pairs of disulfide bond and to exert its stabilizing role through an oxidative folding mechanism [26,27]. It is therefore conceivable that the loss of CXC should affect the stability rather than the flexibility of SsMTAPII. The simultaneous elimination of Cys200–Cys262 disulfide bond and of CXC motif seems to play a synergistic effect on enzyme flexibility. In fact, after 30 min incubation the residual activity of mut3 drops to 42%.

By contrast, the disulfide bond Cys138–Cys205 seems to contribute significantly in maintaining the native conformation of the enzyme. Indeed, after 30 min of proteolysis mut4 appears very degraded by the protease; the primary proteolytic fragment becomes predominant (lane 8 in Fig. 5C) and the catalytic activity drops to 26%, a value similar to that observed in mut6. It is interesting to note that, as evidenced from the three-dimensional structure of SsMTAPII, Cys138 is located in helix α2, a zone of the enzyme involved either in the trimer–trimer interface or in the subunit–subunit interface within a trimer [26]. The loss of the disulfide bridge Cys138–Cys205 could therefore cause a deleterious disturbance of the compact structural organization of the enzyme thus causing increased protease susceptibility.

Finally, the limited proteolysis of mut5, where only C164 is present, gives insights on a possible stabilizing role of Cys164. As indicated by the time course of hydrolysis (Fig. 5B) and by the proteolytic pattern (lane 10 in Fig. 5C), mut5 shows a protease susceptibility more similar to that of mut3 (in which the disulfide bridge Cys138–Cys205 is still present) than that of mut4. It should be noted that Cys164 is located in the strand β9, near the protein surface but not directly exposed to solvent and not involved in the subunit–subunit interface within a trimer [26]. This positioning makes Cys164 to far away from the cysteine residues, either in the same or in the neighboring monomers, to form disulfide bridges. This appears a quite strategic position at high temperatures. In fact, in the presence of disulfide bonds that tether the protein in the compact and rigid native state, the thermolabile amino acid residue Cys164 can be protected from the oxidation. By contrast, when disulfide bonds are damaged or destroyed because of environmental stress, the increased segmental mobility of the polypeptide chain allows Cys164 to perform novel stabilizing disulfide bond(s) with its partner from a neighboring monomer, as demonstrated above by SDS-PAGE under nonreducing conditions (Fig. 4).

In conclusion, the substantial decrease in the values of ΔHcal (850 kcal/mol) and T_m (39 °C) observed by comparing wild-type and mut6 demonstrates that all cysteine pairs, and especially the CXC motif C259–C261, as well as the free C164 significantly contribute to SsMTAPII thermostability.
thermal stability. Furthermore, as deletion of these cysteines does not alter the mutant activities, it can be concluded that they are essential elements involved in structural stability. Moreover, limited proteolysis experiments, in good agreement with the DSC characterization of SsMTAPII, highlight the peculiar stabilization strategy adopted by this hyperthermophilic protein in which two disulfide bonds, a PDI-like CXC motif and a free cysteine residue combine together to maintain the delicate balance between stability and flexibility required by the enzyme to remain stable and functionally active under extreme thermal environments.

Conflict of Interest

All the authors declare no conflict of interest.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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