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Conformational changes and stabilization induced by phosphate binding to 5′-methylthioadenosine phosphorylase from the thermophilic archaeon *Sulfolobus solfataricus*

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Abstract The effect of phosphate, its analogues, and other substrates on structural features of recombinant 5′-methylthioadenosine phosphorylase from *Sulfolobus solfataricus* (SsMTAP) was investigated. Phosphate was found to exert a significant stabilizing effect on the protein against the inactivation caused by temperature, sodium dodecyl sulfate (SDS), urea, and proteolytic enzymes. In the presence of 100 mM phosphate: (i) the apparent transition temperature (T_m) of recombinant SsMTAP increased from 111° to 118°C; and (ii) the enzyme still retained 40% and 30% activity, respectively, after 30 min of incubation at 90°C with 2% SDS or 8 M urea. The structure modification of SsMTAP by phosphate binding was probed by limited proteolysis with subtilisin and proteinase K and analysis of polypeptide fragments by SDS-PAGE. The binding of the phosphate substrate protected SsMTAP against protease inactivation, as proven by the disappearance of a previously accessible proteolytic cleavage site that was localized in the N-terminal region of the enzyme. The conformational changes of SsMTAP induced by phosphate and ribose-1-phosphate were analyzed by fluorescence spectroscopy, and modifications of the protein intrinsic fluorophore exposure, as a consequence of substrate binding, were evidenced.

Key words 5′-Methylthioadenosine phosphorylase · *Sulfolobus solfataricus* · Protein stability · Substrate-induced conformational change · Limited proteolysis

Introduction

Since their discovery, interest in thermophilic microorganisms has expanded rapidly, primarily focusing on understanding the structural and energetic basis of protein stability at extremely high temperatures (Vogt et al. 1997). Elucidation of the mechanisms stabilizing hyperthermophilic proteins not only will provide valuable insight into protein folding and the structure–stability relationships of proteins but also will allow evaluation of strategies that have been proposed to improve protein stability.

Because of its exceptional thermal properties (Cacciapuoti et al. 1994), 5′-methylthioadenosine phosphorylase from *Sulfolobus solfataricus* (SsMTAP), an extreme thermophilic archaeon, can be considered an interesting model to study the mechanisms of protein thermostability and to propose new strategies for improving the stability of enzymes for use in biotechnological processes.

SsMTAP catalyzes the reversible phosphorolysis to free adenine and 5-methylthioribose-1-phosphate (Cacciapuoti et al. 1994) of 5′-methylthioadenosine (MTA), a sulfur-containing nucleoside formed from S-adenosylmethionine by several independent pathways of which the polyamine biosynthesis is quantitatively the most important (Williams-Ashman et al. 1982; Della Ragione et al. 1989).

SsMTAP is a hexameric protein of 160 kDa made up of six identical subunits of 26.5 kDa and is able to cleave, in addition to the 6-amino purine nucleosides MTA and adenosine, also 6-oxo-purine nucleosides, guanosine, and inosine (Cacciapuoti et al. 1994). Therefore, for its broad substrate specificity, SsMTAP could more appropriately be considered a purine nucleoside phosphorylase (PNP) belonging, on the basis of the hexameric structure, to the class of "high molecular mass" phosphorylases (Koellner et al. 1998). Each subunit of SsMTAP contains 236 amino acid residues. Protein sequence analysis of SsMTAP (Cacciapuoti et al. 1996) shows only a relatively low identity with human 5′-methylthioadenosine phosphorylase (MTAP) (Olopade et al. 1995) but a high degree of homology with *Escherichia coli* PNP (Hershfield et al. 1991) and *E. coli* uridine phosphorylase (Walton et al. 1989), which are the homologous enzymes involved in purine and pyrimidine nucleoside metabolism.

SsMTAP is highly thermophilic and extremely thermostable and shows unusual stability in the presence of organic

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solvents, protein denaturants, detergents, and proteolytic enzymes, even at high temperatures (Cacciapuoti et al. 1994). Labeling experiments demonstrated the presence in the protein of six disulfide bonds that are probably positioned as intersubunits, resulting in the organization of the enzyme into two trimers (Cacciapuoti et al. 1994). These covalent links, in addition to the number of possible weak intramolecular interactions, could be responsible for the extreme thermal stability of SsMTAP. It is interesting to note that the presence of multiple disulfide bonds, which is not a common event in intracellular proteins and has been reported only in a few cases (Toth et al. 2000), could represent one of the strategies utilized by hyperthermophilic proteins to reach higher levels of stability.

SsMTAP has been expressed with high yield in *E. coli* (Cacciapuoti et al. 1999). The recombinant enzyme is identical to the original SsMTAP in its physicochemical and kinetic properties but it is less thermophilic and thermostable because an incorrect positioning of disulfide bonds within the molecule generates structures less stable to thermal unfolding (Cacciapuoti et al. 1999).

As previously reported for wild-type SsMTAP phosphate, one of the substrates of the reaction, and its analogue sulfate, exert a significant protection against thermal inactivation (Cacciapuoti et al. 1994). Moreover, kinetic data (Kierdaszuk et al. 1997) and crystallographic studies (Koellner et al. 1998) support the hypothesis of the presence in *E. coli* PNP of two enzyme conformations with different affinities for phosphate.

To evaluate the stabilizing effect of phosphate on recombinant SsMTAP and to determine if this effect results from a specific interaction with the enzyme or has to be ascribed to an aspecific salt effect, we have investigated how the presence of phosphate, of some of its analogues, and of other reaction substrates can affect the stability of the enzyme. Moreover, we showed by means of limited proteolysis that the binding of phosphate alters the sensitivity of the protein to digestion, indicating that SsMTAP undergoes stabilizing conformational changes under these conditions. Finally, we demonstrated the conformational changes induced by phosphate and ribose-1-phosphate binding by fluorescence spectroscopy.

Materials and methods

Chemicals

5′-Methylthioadenosine (MTA) and 5′-[*methyl*-^{14}C]MTA were prepared from unlabeled and labeled S-adenosylmethionine and purified by HPLC (Cacciapuoti et al. 1994). Proteinase K, subtilisin, phenylmethylsulfonyl fluoride (PMSF), ribose-1-phosphate, and standard proteins used in molecular mass determinations were from Sigma (St. Louis, MO, USA). Polyvinylidene fluoride (PVDF) membranes (0.45 mm pore size) were obtained from Millipore (Bedford, MA, USA). All reagents were of the purest commercial grade.

Protein and assay

The recombinant SsMTAP was overexpressed and purified as described previously (Cacciapuoti et al. 1999). MTA phosphorylase activity was determined by measuring the formation of [*methyl*-^{14}C]5-methylthioribose-1-phosphate from [*methyl*-^{14}C]MTA, as described by Cacciapuoti et al. (1994). Protein concentration was estimated according to Bradford (1976) using human γ-globulin as standard.

Limited proteolysis and protein sequence analysis

Proteolytic inactivation of recombinant MTAP by proteinase K and subtilisin was carried out in 20 mM Tris-HCl, pH 7.4, at 37°C. The final mass ratio of substrate protein to protease was 20 : 1. At different incubation times the hydrolysis was stopped by the addition of phenylmethylsulfonylfluoride (PMS) (final concentration, 250 μM) and the samples were assayed for MTA phosphorylase activity. The digested material was submitted to electrophoresis on sodium dodecyl sulfate-containing 15% polyacrylamide gels followed by staining with Coomassie blue R-250.

For the amino acid sequence analysis, samples of the digested recombinant MTAP, after sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE), were electrophoretically blotted onto a PVDF 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 10 min at room temperature, and allowed to air dry. Stained protein bands were excised from the blot and their NH_2-terminal sequences were determined.

Stability and thermostability studies

The stability of recombinant MTAP activity in the presence of urea and SDS was tested at the indicated temperatures by incubating, in stoppered glass tubes, the enzyme (40 μg) and the compound to be tested in 20 mM Tris-HCl, pH 7.4, in a final volume of 100 μl. Immediately after the addition of the compound (time-zero control) and at different time intervals, 5-μl aliquots were removed from each sample and the enzyme activity was assayed as described. Activity values are expressed as percentage of the time-zero control (100%). Enzyme thermostability was tested by incubating the protein in sealed glass vials at temperatures between 90° and 125°C. Activity was assayed in standard conditions by the addition of a 20-μl aliquot (2 μg) from each preincubated sample.

Fluorescence spectroscopy

Fluorescence measurements were carried out on a Perkin-Elmer (Norwalk, CT, USA) MPF-44 spectrofluorometer in the range of fluorescence linearity. The absorbance of all solutions was 0.05–0.10 at the excitation wavelength. The

excitation and emission band widths were set at 5 nm. In the fluorescence quenching experiments, the measurements, which were corrected for the dilution effect and for background fluorescence of the buffer, were made at the controlled temperature of 20°C. The excitation wavelength was 295 nm. After each addition to the sample of the titrating 1 M KI solution containing 0.1 mM potassium thiosulfate, the fluorescence was compared to that of an equivalent sample added with a KCl solution at the same concentration of KI. Except for dilution, no effect of KCl addition on protein fluorescence was observed. When the quenching data, plotted according to the Stern–Volmer equation $F_o/F = 1 + K_{sv}[Q]$, showed a downward curvature indicating heterogeneous quenched fluorophores, they were plotted according to the equation:

$$F_o/(F_o - F) = 1/(f_a K_{sv}[Q]) + 1/f_a$$

where F_o is the fluorescence in the absence of the quencher Q, f_a is the fraction of protein-quenchable fluorescence, and K_{sv} is the Stern–Volmer constant (Lakowicz 1983).

Results and discussion

Stabilizing effect of phosphate on enzyme thermostability

Although highly thermophilic and thermostable, recombinant SsMTAP is less thermophilic and thermostable than the wild-type enzyme (Cacciapuoti et al. 1999). Nonetheless, SsMTAP and recombinant SsMTAP share the same quaternary structure, kinetic constants, and substrate specificity, suggesting that the active site is identical and that the overall structural organization is very similar (Cacciapuoti et al. 1999).

To evaluate the possible stabilizing effect of phosphate substrate on the thermostability of recombinant SsMTAP, we measured the melting temperature of the enzyme and carried out the long-term kinetics of thermal inactivation at 100°C in both the presence and absence of 100 mM sodium phosphate. As shown in Fig. 1a, phosphate exerts a protection toward temperature inactivation of recombinant SsMTAP, which after 1 h incubation at 100°C retains 95% of its catalytic activity in comparison with 82% activity measured when the enzyme is preincubated alone. Figure 1b shows the diagram of the residual activity after 10 min of preincubation as a function of temperature, from which a transition temperature (apparent T_m) of 111°C was calculated. This value increases to 118°C in the presence of 100 mM phosphate, thereby indicating that the binding of this substrate increases the conformational stability of the enzyme, thus modifying its susceptibility to thermal denaturation.

Stabilization against SDS and urea by substrates and analogues

Recombinant SsMTAP is stable at room temperature in the presence of 2% SDS but becomes completely unstable in the presence of this detergent at 70°C. Figure 2a shows the protective effect exerted by increasing concentrations of sodium phosphate on the stability of recombinant SsMTAP in the presence of 2% SDS at 70°C. The stabilizing effect appears to be a function of phosphate concentration and reaches the maximal value at 100 mM phosphate, at which point the enzyme, after 1 h incubation, still retains 98% activity. In contrast, in the same experimental conditions but in the absence of phosphate, the enzyme is completely inactive without preincubation.

Phosphate binding is also able to increase the already high stability of the wild-type enzyme toward the detergent. In fact, after 1 h incubation at 90°C with 100 mM sodium phosphate and 2% SDS, the residual activity of the wild-type enzyme increases from 20% to 60% (data not shown). These data indicate that, although differing in their stability, wild-type and recombinant MTAP show the same behavior in the presence of phosphate, further confirming the identity of the overall structural organization.

Figure 2b compares the SDS-PAGE patterns obtained by incubating, for 5 min at 70°C, recombinant SsMTAP with

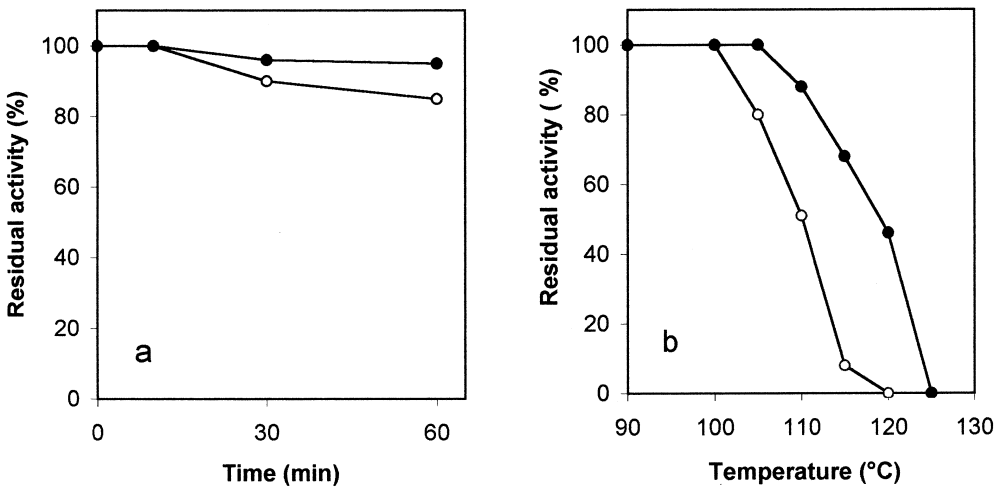

Fig. 1a,b. Thermostability of recombinant 5′-methylthioadenosine phosphorylase from *Sulfolobus solfataricus* (SsMTAP). **a** Kinetics of thermal inactivation at 100°C as a function of incubation time in the presence (*solid circles*) and in the absence (*open circles*) of 100 mM phosphate. **b** Residual 5′-methylthioadenosine (MTA) phosphorylase activity after 10 min of incubation at temperatures shown in the presence (*solid circles*) and in the absence (*open circles*) of 100 mM phosphate

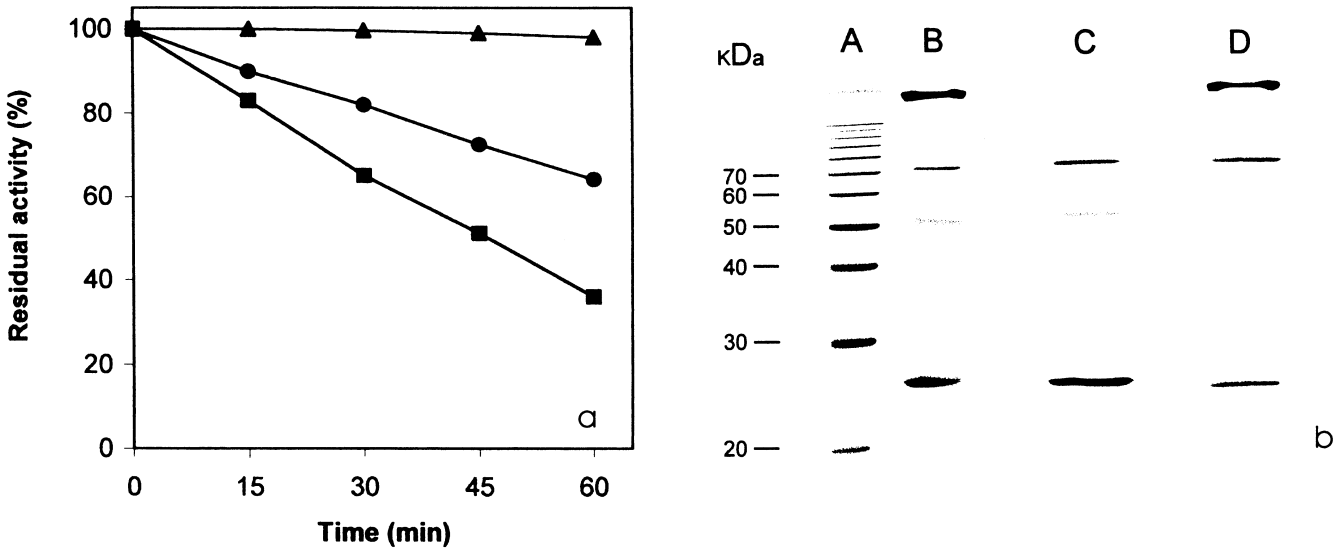

Fig. 2a,b. Effect of phosphate concentration on the stability of recombinant SsMTAP in the presence of 2% sodium dodecyl sulfate (SDS). **a** Samples containing the enzyme were incubated in 20 mM Tris-HCl, pH 7.4 containing 2% SDS at 70°C in the presence of 35 mM (*squares*), 50 mM (*circles*), and 100 mM (*triangles*) sodium phosphate. At the time indicated, aliquots were withdrawn and assayed for MTA phosphorylase activity as described in Materials and methods. Activity values are expressed as percentage of the time-zero control (100%). **b** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of recombinant SsMTAP incubated for 5 min at 70°C. The samples were subjected to SDS-PAGE without boiling and under non-reducing conditions. *Lane A*, molecular mass markers; *lane B*, recombinant SsMTAP (5 μg); *lane C*, recombinant SsMTAP incubated with 2% SDS; *lane D*, recombinant SsMTAP incubated with 2% SDS plus 100 mM phosphate

2% SDS in the presence or absence of 100 mM phosphate. The samples were subjected to SDS-PAGE without boiling and under nonreducing conditions to obtain a picture of the protein species present in the experimental conditions described. In lane B is depicted the complex electrophoretic pattern of the recombinant enzyme incubated in the absence of both 2% SDS and 100 mM phosphate. In addition to the protein band at 160 kDa, which corresponds to the hexamer molecular mass, this lane shows several other protein species; the protein band at 27 kDa is the monomer of the enzyme, whereas the protein bands at about 50 and 80 kDa should correspond to species with a different structural organization, probably generated by the incorrect positioning of disulfide bonds between the subunits of the enzyme, as previously demonstrated (Cacciapuoti et al. 1999). The presence of 2% SDS causes the complete loss of both MTA phosphorylase activity and the protein band at 160 kDa (lane C), suggesting that the hexamer is the only active molecular species. In contrast, the presence of 100 mM phosphate completely counteracts the effect of the detergent, stabilizing the hexameric active SsMTAP structure (lane D).

To check the effect of phosphate on the thermostability of recombinant SsMTAP against detergents and denaturants, the enzyme was incubated at 70°, 80°, and 90°C with 2% SDS in the presence of 100 mM sodium phosphate, and with 8 M urea in the presence and in the absence of the anion (Fig. 3). In the presence of phosphate, after 1 h incubation with 2% SDS the enzyme still retains 75% and 32% residual activity at 80° and 90°C, respectively (Fig. 3a). Furthermore, in the presence of phosphate, the enzyme remains completely active after 1 h incubation with 8 M urea at 70°C and still retains 30% residual activity after 30 min at 90°C (Fig. 3b).

Kosmotropes salts such as phosphate and sulfate are known to stabilize many proteins through general electrostatic and hydrogen-bonding effects on the protein and on the solvent (Low et al. 2000). Because phosphate is a substrate of MTAP, it is possible that the stabilization observed in the presence of this anion could be ascribed to its specific interaction with the enzyme. To verify this hypothesis, we checked the effect of salts and substrates on the stability of recombinant enzyme in the presence of 2% SDS at 70°C (Table 1).

Sulfate and arsenate act as inhibitor and substrate, respectively, of the thermophilic enzyme, as they do for *E. coli* PNP (Jensen and Nygaard 1975) and calf spleen PNP (Kline and Schramm 1993). They stabilize the enzyme toward denaturation even if at a lower extent than phosphate. It is interesting to note that, because of its close similarity with the phosphate anion, the arsenate anion is a more potent stabilizing agent than sulfate. The lack of any effect exerted by sodium perchlorate and sodium chloride strongly supports the view of a specific interaction of phosphate and its analogues with the enzyme. Among the other substrates of the reaction, only ribose-1-phosphate, at a concentration much lower than phosphate, is able to protect the enzyme against the detergent, whereas adenine and MTA are completely ineffective. These data suggest that ribose-1-phosphate establishes with the enzyme a stronger interaction than phosphate, and that MTA and adenine, in analogy with *E. coli* PNP (Koellner et al. 1998; Jensen 1976), form

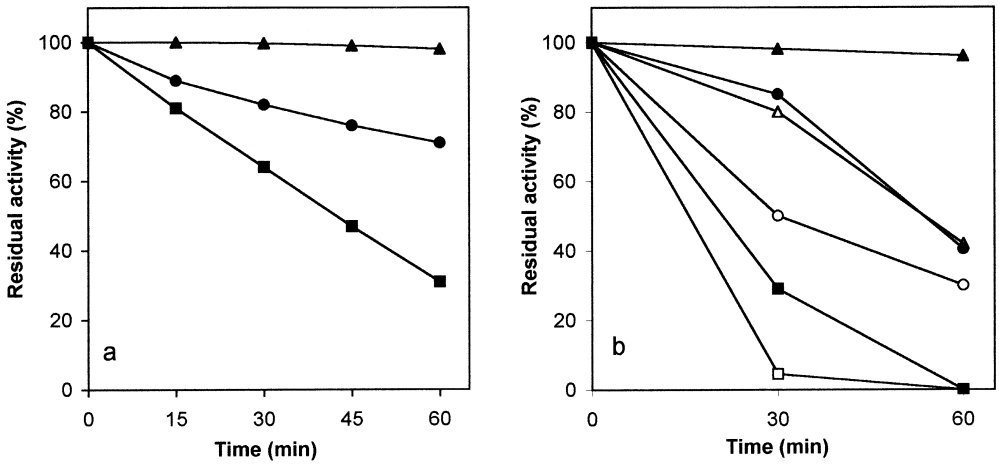

Fig. 3a,b. Effect of phosphate on the thermostability of recombinant SsMTAP in the presence of 2% SDS and 8 M urea. **a** The enzyme was incubated with 2% SDS in the presence of 100 mM phosphate at 70°C (*solid triangles*), 80°C (*solid circles*), and 90°C (*solid squares*). **b** The enzyme was incubated with 8 M urea at 70°C with (*solid triangles*) or without (*open triangles*) 100 mM phosphate; at 80°C with (*solid circles*) or without (*open circles*) 100 mM phosphate; and at 90°C with (*solid squares*) or without (*open squares*) 100 mM phosphate. At the time indicated, aliquots were withdrawn and assayed for MTA phosphorylase activity as described in Materials and methods. Activity values are expressed as percentage of the time-zero control (100%)

Table 1. Stabilizing effect of substrates and salts on the stability of recombinant 5′-methylthioadenosine phosphorylase from *Sulfolobus solfataricus* (SsMTAP) in the presence of 2% SDS at 70°C

Compound	Residual activity (%)	
	30 min	60 min
None	n.d.	n.d.
5 mM MTA	n.d.	n.d.
5 mM adenine	n.d.	n.d.
5 mM ribose-1-phosphate	62.4	44.6
100 mM sodium phosphate	100	98
100 mM sodium arsenate	58	51
100 mM sodium sulfate	28	20
100 mM sodium perchlorate	n.d.	n.d.
100 mM sodium chloride	n.d.	n.d.

Activity values are expressed as percentage of the time-zero control (100%) (see Materials and methods)
MTA, 5′-methylthioadenosine; n.d., not detectable

with the enzyme only ternary complexes in the presence of phosphate and ribose-1-phosphate, respectively.

The presence of phosphate ions alters the accessibility of proteolytic target sites

The application of limited proteolysis can often provide useful information about conformational changes resulting from the interaction of a protein with substrate or effector molecules. The susceptibility of a protein region to proteolytic cleavage can be modified because of a conformational change that results either in protection of the cleavage sites or in uncovering new sites (Kampranis and Maxwell 1998; Lindsley and Wang 1993).

By comparing the proteolytic pattern of recombinant SsMTAP in the presence or in the absence of phosphate, it has been possible to detect conformational changes associated with this interaction. Because recombinant SsMTAP, like the wild-type enzyme (Cacciapuoti et al. 1994), is resistant to the proteolytic degradation by several mesophilic (trypsin, chymotrypsin) and thermophilic (thermolysin) proteases, we selected two endoproteases of broader substrate specificity, i.e., proteinase K and subtilisin. The time-course for the hydrolysis of recombinant SsMTAP with proteinase K (Fig. 4a) and subtilisin (Fig. 4c) followed by SDS-PAGE (Fig. 4b and Fig. 4d, respectively) shows that in both cases a protein band with an apparent molecular mass about 0.8 kDa less than that of recombinant SsMTAP appears as the catalytic activity decreases, suggesting that the cleavage of the protein by both proteases occurred at the same specific site. After 4 h incubation at 37°C with proteinase K, that protein band becomes the most abundant and the activity drops to 10% (Fig. 4a), whereas, in the presence of subtilisin, a higher level of resistance to the proteolytic process is evident and the enzyme still retains 24% activity after 6 h incubation (Fig. 4c).

To identify the cleavage site, the N-terminal amino acid sequence of the proteic band of lower molecular mass obtained after the proteolytic digestion with the two proteases was determined. The obtained sequences showed that both fragments started at Ala-8, indicating that the cleavage site was the Leu-7–Ala-8 bond. When the experiment was carried out in the presence of 100 mM phosphate, the enzyme remained completely active and the proteolytic process did not occur (see lane G in Fig. 4e). This protection against proteolysis provides the first direct evidence of a phosphate-induced conformational change in the recombinant SsMTAP. Moreover, these results indicate that the amino-terminal peptide is necessary for the integrity of the active site, in analogy with that observed for *E. coli* PNP, where His-5 was found to be involved in the binding with

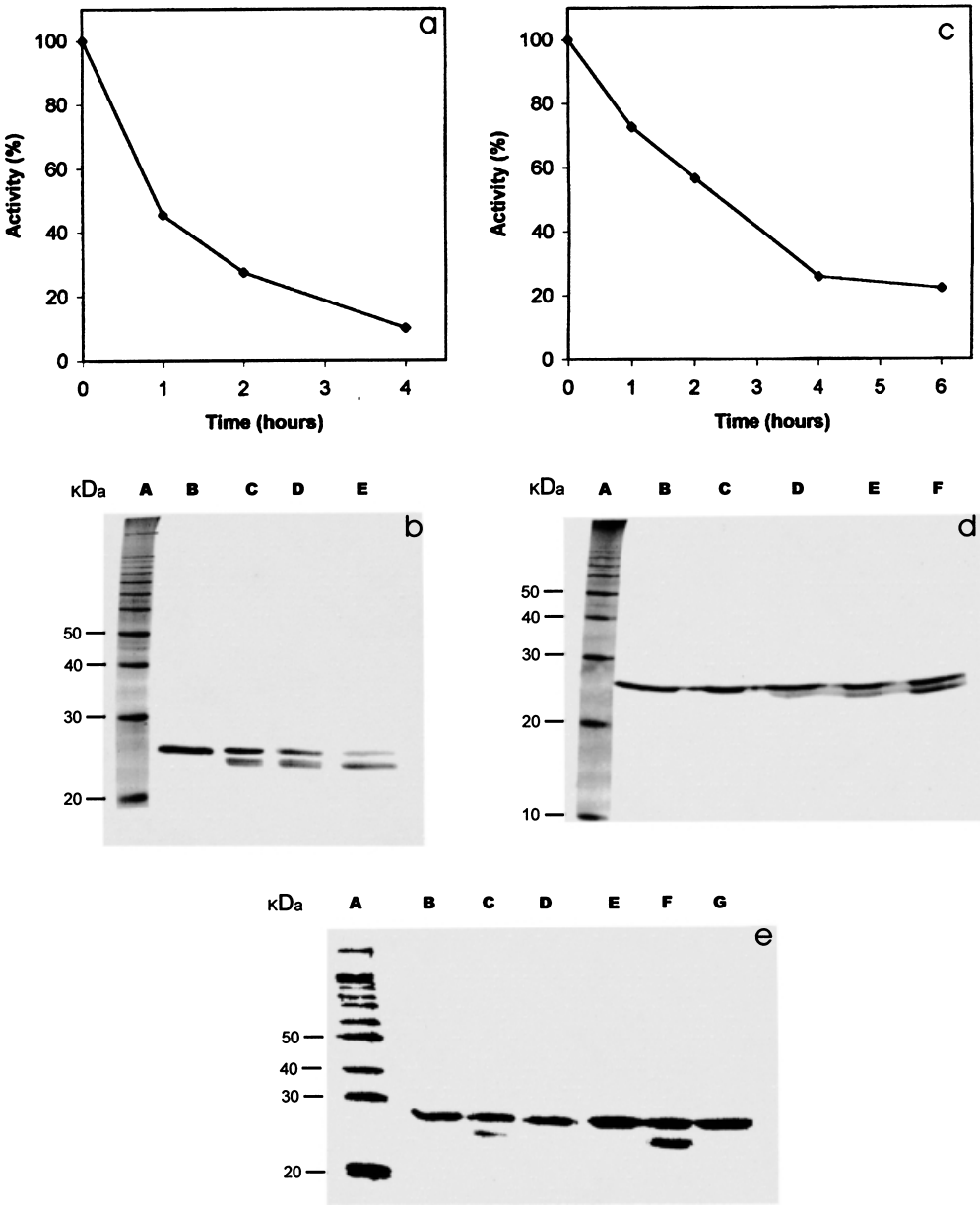

Fig. 4a–e. Limited proteolysis of recombinant and wild-type SsMTAP. Time-course for the hydrolysis of recombinant SsMTAP with proteinase K (**a**) and subtilisin (**c**) followed by SDS-PAGE (**b** and **d**, respectively). The final mass ratio of SsMTAP to protease was 20 : 1. Aliquots of recombinant SsMTAP at different incubation times at 37°C with proteases were taken from the reaction mixture and the hydrolysis was stopped (see Methods). Samples were assayed for 5′-methylthioadenosine phosphorylase (MTAP) activity at 70°C, subjected to SDS-PAGE, and gels stained with Coomassie brilliant blue. **e** SDS-PAGE of wild-type and recombinant SsMTAP. *Lanes A*, molecular mass markers; *lanes B–D*, wild-type SsMTAP (2 µg) after 0 h (*B*) and 1 h incubation in the absence (*C*) and in the presence (*D*) of 100 mM phosphate. *Lanes E–G*, recombinant SsMTAP (8 µg) after 0 h (*E*) and 1 h incubation in the absence (*F*) and in the presence (*G*) of 100 mM phosphate

ribose (Koellner et al. 1998). In this respect, it is interesting to note that all the amino acid residues involved in the active site of *E. coli* PNP, identified on the basis of crystallographic data (Koellner et al. 1998), are completely conserved in the thermophilic enzyme with the exception of Ser-91 of *E. coli* PNP, which is substituted by a threonine in SsMTAP. This observation extends the analogies between SsMTAP and *E. coli* PNP from the structure to the mechanism of action of these enzymes.

To verify whether phosphate exerts on wild-type SsMTAP the same protective effect against proteolysis as observed with recombinant SsMTAP, the enzyme has been subjected to a proteolytic digestion with proteinase K in the absence and in the presence of 100 mM phosphate (Fig. 4e). The results obtained indicate that only one fragment is produced by the action of protease on wild-type SsMTAP (lane C), of the same molecular mass of that obtained in the same experimental conditions with the recombinant form of the enzyme (lane F). Furthermore, similar to recombinant SsMTAP (lane G), the binding of phosphate to the wild-type enzyme masks the proteolytic site (lane D), thus indicating a conformational protein change in this case also.

Substrate-induced conformational changes analyzed by fluorescence spectroscopy

Unlike the homologue enzyme *E. coli* PNP, which lacks tryptophanyl residues (Hershfield et al. 1991), SsMTAP possesses three tryptophanyl residues per subunit that are clustered in the C-terminal region at positions 171, 195, and 213 (Cacciapuoti et al. 1996). The fluorescence emission spectra of the protein in Tris buffer, phosphate buffer, and Tris buffer containing ribose-1-phosphate are reported in

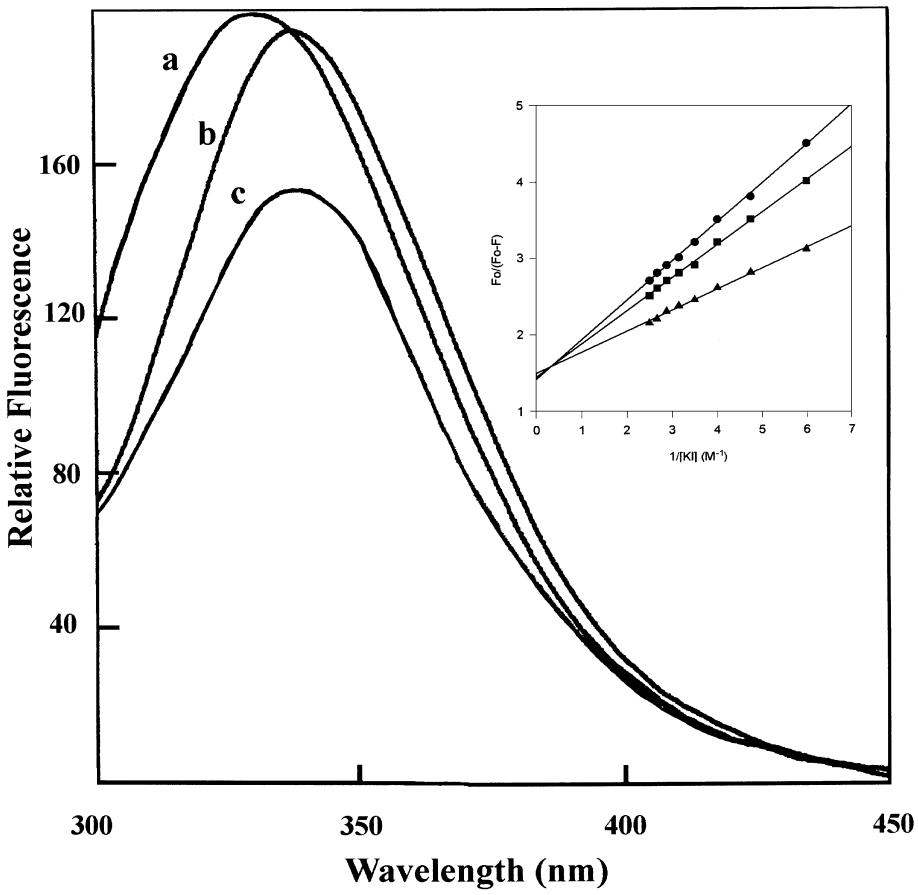

Fig. 5. Fluorescence emission spectra of recombinant SsMTAP. Spectra of recombinant SsMTAP in the presence of 100 mM Tris-HCl (*a*), 100 mM phosphate (*b*), and 100 mM Tris-HCl plus 5 mM ribose-1-phosphate (*c*). Excitation was at 280 nm. The spectra were corrected for blanks represented by the same solutions without the protein; pH was 7.4 and protein concentration was 1×10^{-6} M. *Inset:* modified Stern–Volmer plot. Iodide quenching of the enzyme was monitored in 100 mM Tris buffer (*triangles*), in 100 mM Tris buffer plus 5 mM ribose-1-phosphate (*circles*), and in 100 mM phosphate buffer (*squares*). The excitation was at 295 nm and the emission was recorded at 340 nm. The *straight lines* interpolating the data were obtained with the least-squares method

Fig. 5. The emission maximum of the protein is centered at 337 nm and undergoes a slight red shift of about 5 nm in the presence of both phosphate and ribose-1-phosphate. Moreover, the fluorescence intensity decreases about 5% and 25% in the presence of phosphate and ribose-1-phosphate, respectively.

The different spectra observed indicate substrate-induced structural protein changes leading to the variation of the mean exposure to the solvent of the tryptophanyl residues. This effect has been confirmed by fluorescence quenching experiments performed with potassium iodide. The Stern–Volmer plots obtained nonlinear results, showing, in all cases, a downward curvature indicative of a different accessibility of the iodide ions to the fluorophores. Plotting the data according to the modified form of the Stern–Volmer equation (see Materials and methods), three straight lines were obtained, with similar ordinate intercepts but with different slopes (see inset in Fig. 5). The Stern–Volmer constants for the protein in the presence of ribose-1-phosphate, phosphate, and Tris buffer were 2.78 M^{-1}, 3.38 M^{-1}, and 5.54 M^{-1}, respectively. Superimposable results have been obtained when wild-type SsMTAP was subjected to the same fluorescence analysis, indicating that, in the presence of phosphate or ribose-1-phosphate, the environment of the fluorophores undergoes a structural modification.

In conclusion, in this article we have demonstrated, by activity assays, limited proteolysis experiments, and fluorescence spectroscopy, that the binding of phosphate and ribose-1-phosphate to SsMTAP induces a conformational transition that stabilizes the folded structure of the enzyme. Because protein flexibility is fundamental in the proteolytic event, the application of limited proteolysis has provided, in the absence of the X-ray structure, useful information about the SsMTAP flexible regions exposed to the solvent and susceptible to proteolytic attack. Experiments of limited proteolysis demonstrated that the enzyme shows a cleavage site exposed to proteolytic digestion in its amino terminal region and that the amino terminal peptide is necessary for the integrity of the active site as its hydrolysis results in enzymatic inactivation.

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