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A novel thermostable sulfite oxidase from *Thermus thermophilus*: characterization of the enzyme, gene cloning and expression in *Escherichia coli*

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Abstract A novel sulfite oxidase has been identified from *Thermus thermophilus* AT62. Despite this enzyme showing significant amino-acid sequence homology to several bacterial and eukaryal putative and identified sulfite oxidases, the kinetic analysis, performed following the oxidation of sulfite and with ferricyanide as the electron acceptor, already pointed out major differences from representatives of bacterial and eukaryal sources. Sulfite oxidase from *T. thermophilus*, purified to homogeneity, is a monomeric enzyme with an apparent molecular mass of 39.1 kDa and is almost exclusively located in the periplasm fraction. The enzyme showed sulfite oxidase activity only when ferricyanide was used as electron acceptor, which is different from most of sulfite-oxidizing enzymes from several sources that use cytochrome *c* as co-substrate. Spectroscopic studies demonstrated that the purified sulfite oxidase has no cytochrome like domain, normally present in homologous enzymes from eukaryotic and prokaryotic sources, and for this particular feature it is similar to homologous enzyme from *Arabidopsis thaliana*. The identified gene was PCR amplified on *T. thermophilus* AT62 genome, expressed in *Escherichia coli* and the recombinant protein identified and characterized.

Keywords Sulfite oxidase · *Thermus thermophilus* · Thermophilic enzyme · Molybdenum cofactor · Ferricyanide

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Introduction

Sulfite oxidases (SO), the enzymes that catalyze the two electrons oxidation of sulfite to sulfate ($SO_3^{2-} + H_2O \rightarrow SO_4^{2-} + 2H^+ + 2e^-$), are molybdenum enzymes belonging to the "Sulfite oxidase" family that comprises sulfite-oxidizing enzymes and plant assimilatory Nitrate reductase. In all known cases, these enzymes possess a molybdenum cofactor (MoCo) characterized by the presence of a mononuclear Mo atom coordinated to the sulfur atoms of a pterin derivative, named molybdopterin, and to the protein by a cysteine residue that is conserved within the family (Hille 1996).

Until now SO has been isolated from eukaryotes such as man (Cohen and Fridovich 1971a, b), mouse (Garrett and Rajagopalan 1994), and chicken (Kisker 2001; Kessler and Rajagopalan 1972) liver and plant *Arabidopsis thaliana* (Eilers et al. 2001), and from prokaryotes such as *Thiobacillus novellus* (Toghrol and Southerland 1983; Kappler et al. 2000), reclassified as *Starkeya novella* (Kelly et al. 2000), *Thiobacillus thioparus* (Lyric and Suzuki 1970), and *Thiobacillus acidophilus* (de Jong et al. 2000). Although SO is ubiquitous among different sources, there is a great variability in the enzyme characteristics with respect to the cellular localization, molecular mass, catalytic parameters and redox centre type. Sulfite oxidases from vertebrate sources are the best studied among the molybdenum enzymes. SOs from these sources are localized in the intermembrane space of the mitochondria and are homodimers of 110 kDa molecular mass consisting of three functional domains: an N-terminal heme domain, a MoCo, and a C-terminal dimerization domain (Kisker 2001). Chicken SO has been crystallized (Kisker et al. 1997) and mammalian enzymes have also been studied by electron paramagnetic spectroscopy (EPR) (Eilers et al. 2001, Kappler et al. 2000; Bray et al. 1983), and resonance Raman spectroscopy (Raitsimring et al. 1998; Johnson et al. 1997) and the catalytic mechanism has been established (Hille 1996; Brody and Hille 1999).

Recently a c-DNA was isolated from *Arabidopsis thaliana* with high homologies with the MoCo domain of the chicken SO (Eilers et al. 2001). This enzyme is different from the mammalian SO, showing only the MoCo domain as the redox centre and is localized in the peroxisomal space. The lack of the prosthetic heme group has been shown not only by the amino acid sequence but also by spectral and enzymatic properties. A reasonable assumption on in vivo function of this enzyme is that in plants the SO electron acceptor could be taken over by a large number of other molecules (Eilers et al. 2001). Moreover, crystallographic evidence suggests that where present the heme domain is very far (32 Å) from the MoCo domain and that they are very mobile, being clearly separated by a long and flexible hinge, indicating that the covalent bond is not necessary for the correct electron transfer (Pacheco et al. 1999).

Several microorganisms including Bacteria and Archaea are able to use sulfite as sole electron source for chemotrophic, lithotrophic and phototrophic growth (Friedrich 1998; Brune 1995; Kelly et al. 1997). Two pathways for sulfite oxidation are known: direct oxidation to sulfate catalyzed by a sulfite oxidase, and indirect AMP depending oxidation (Brüser et al. 2000; Taylor 1994; Bick et al. 2000). The simultaneous presence of both sulfite oxidation pathways has been identified for a large number of sulfur oxidizing bacteria (e.g., *Thiobacillus denitrificans*, *Thiobacillus thioparus*, *Allochromatium vinosum*) as reviewed in Friedrich 1998, the green sulfur bacteria (Trüper and Fisher 1982), the Gram-positive bacteria (genus *Sulfobacillus*) (Krasil'nikova et al. 1998) and the archaeon *Acidianus ambivalens* (Zimmermann et al. 1999). Nevertheless, the direct oxidation of sulfite occurs as means of detoxification and the best studied enzymes belong to *Thiobacillus* group (Kappler and Dahl 2001). The presence of neutrophilic and acidophilic organisms in this group has important implication for the localization and the catalytic properties of the enzymes. The best characterized enzyme is a sulfite:cytochrome *c* oxidoreductase isolated from *Thiobacillus novellus* (Toghrol and Southerland 1983; Kappler et al. 2000). In this organism the enzyme is a $\alpha\beta$ heterodimer located in the periplasm, constituted by a 40.6 kDa subunit containing MoCo and by a 8.8 kDa cytochrome c552-like subunit: it exhibits kinetic and inhibition properties very similar to eukaryotic SOs (Brody and Hille 1999).

Bacteria of the genus *Thermus* have been isolated from many natural and artificial thermal environments throughout the world (Brock and Freeze 1969; Munster et al. 1986; Hudson et al. 1987; Saiki et al. 1972). The taxonomic position of *Thermus* relative to other genera remains uncertain, but an oligonucleotide catalog of 16S rRNA has indicated a remote but distinct relationship between *T. aquaticus* and *T. ruber* and *Deinococcus* (Hensel et al. 1986).

No data about sulfur metabolism in *T. thermophilus* is available in the literature, but some information can be deduced from the organization of the genome of *T. thermophilus* HB27 which has been recently sequenced and made available (Henne et al. 2004). The identification of genes encoding molybdopterin containing enzymes (such as a cluster containing several genes homologous to the SO genes of many sulfur-oxidizing organisms) would suggest that cells are able to oxidize sulfite. Function assignment by characterization of the gene products after cloning and heterologous expression would definitively help in the definition of this specific metabolic feature of *T. thermophilus*.

The AT 62 strain has an optimum growth temperature of 75°C, and its enzymes have a broad temperature range of activity. This strain of the genus *Thermus* is source of several restriction endonucleases that are both thermostable and active at higher temperatures than most other such enzymes.

Here we report the purification and the characterization of the first sulfite oxidase from a thermophilic microorganism, i.e., *T. thermophilus* AT 62 (ATCC 33923 previously designated *Thermus flavus*). The native enzyme (Tf-SO) was purified and characterized and the specific search on the *T. thermophilus* HB27 genome revealed an open reading frame (ORF) encoding a putative sulfite oxidase (Tf-SO2) with the predicted gene product showing significant similarity to corresponding proteins already characterized. The sequence was cloned and heterologously expressed in *E. coli* and the enzymatic characterization of its gene product performed in comparison with the specific wild type protein in *T. thermophilus* cells.

Experimental

Bacteria, plasmids, enzymes and chemicals

E. coli pMos*Blue* cells were used for plasmid propagation; TP1000 (*Δ*mobAB) (Temple et al. 2000) strains kindly provided by Dr Tracy Palmer (Department of Molecular Microbiology, John Innes Centre, Norwich), was the hosts for gene expression. All strains were routinely cultured in Luria-Bertani medium, with ampicillin (100 μg/ml).

Thermus thermophilus strain AT62 (DSMZ 674) was grown in modified DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) medium 74 containing 8 g/l tryptone, 4 g/l yeast extract, 1 g/l glucose, 2 g/l NaCl, final pH 7.0–7.2 (medium A).

Cells were harvested by centrifugation (Sorvall RC5C, 6,090×*g*, 15 min, 4°C), recovering about 2 g/l of wet cell. The pellet was stored at −80°C when not immediately used.

Several concentrations of sodium molybdate (0.083, 0.41, 2.07 mM) were added to medium A to increase the SO expression.

The SO purification was carried out using cells grown in medium A supplemented with 2.07 mM sodium molybdate.

Plasmid vectors pGEM®-T Easy and pQE-60 were supplied by Promega and Qiagen, respectively.

Restriction/modification enzymes and Taq DNA polymerase were obtained from New England Biolabs and Finnzymes respectively.

IPTG, X-Gal, all buffers, organic solvents and reagents, were purchased from Oxoid, Sigma, Fluka and ICN in per analisi or corresponding quality.

SO purification

Except where otherwise stated, all purification steps were performed at 4°C. 80 g of frozen *T. thermophilus* cell material were homogenized (Ultra turrax T18 Basic, IKA WORKS, USA) in 10 mM Tris/HCl pH 8.0 and cells broken by three passes through a French pressure cell at about 147 MPa. Cell debris was removed by ultracentrifugation (Beckman L8-70 M Ultracentrifuge: 1 h, 114,000×*g*, 4°C). The supernatant was subjected to ammonium sulfate precipitation (60% saturation) and centrifugation. The precipitate was then dissolved in 70 ml of extraction buffer and dialyzed against 2×5 l of the same buffer. The dialyzed extract (73 ml), corresponded to the crude extract, was applied to a DEAE column (DEAE 52, Whatman) (5×32 cm), equilibrated in the same buffer, and eluted using a linear sodium chloride increasing gradient (1,000 ml, 0–400 mM NaCl).

Active fractions (75 ml), were pooled and concentrated by ultrafiltration (Millipore, PM10). The retentate (3 ml) was gel filtered on Superdex XK26 (Amersham Pharmacia Biotech) equilibrated in 10 mM Tris/HCl pH 8.0 containing 150 mM NaCl. The SO activity exhibiting fractions were pooled and extensively dialyzed against 2×5 l of 10 mM Tris/HCl pH 8.0. Active pool (13 ml) was adsorbed into Hydroxyapatite column (HTP Biogel, Biorad, 4.5×10.5 cm) equilibrated in 10 mM Tris/HCl pH 8.0, and then eluted using a linear potassium phosphate buffer (400 ml, 10–500 mM Potassium phosphate pH 7.6). Finally, active pool (35 ml), dialyzed against 10 mM Tris/HCl pH 8.0, were applied to a Mono Q HR 5/5 (Amersham Pharmacia Biotech) column, equilibrated in the same buffer and the protein eluted by a linear sodium chloride gradient (60 ml, 0–200 mM NaCl). The fractions exhibiting SO activity (2 ml) were concentrated by ultrafiltrations (Centricon YM 10 kDa cut-off, Millipore) until 0.250 ml and checked for purity by SDS-PAGE.

SO activity measurement

Sulfite oxidase was routinely assayed by monitoring reduction of ferricyanide (ε $K_3Fe(CN)_6$ 420 nm = 1.02×10^3 M^{-1} cm^{-1}). The standard reaction mixture contained 0.4 mM potassium ferricyanide in 100 mM Tris/HCl pH 8.0, 0.1 mM EDTA, and 0.4 mM sodium sulfite. The reaction was carried out at 60°C and was started by the addition of enzyme. The rate of reaction was quantified in a Cary 100 (Varian) spectrophotometer by monitoring the decreasing in absorbance at 420 nm. All activity determinations were taken as the mean of three measurements and were corrected for non-enzymatic sulfite oxidation.

One unit of SO activity was defined as the conversion of 1 μmol of sulfite per minute at 60°C.

Cytochrome *c* reductase activity was assayed as described (Cohen and Fridovich 1971a, b).

Steady-state dependence of catalysis on sulfite was conducted in these conditions, using a saturating concentration of ferricyanide (0.400 mM) and varying the concentration of sulfite between 5 and 400 μM. The study of dependence of catalysis on ferricyanide concentration was conducted in the same conditions, using a saturating concentration of sulfite (0.400 mM) and varying the concentration of ferricyanide between 5 and 400 μM (0.300 μg of SO was used for each assay).

The method of Cohen and Fridovich (1971a, b) was used for SO activity staining on native PAGE.

Preparation of spheroplasts

For spheroplast formation, cells were harvested by centrifugation for 20 min at 21,000×*g* (4°C). Cells (0.5 g/ml) were suspended in ice-cold 10 mM Tris/HCl (pH 8.0) and were centrifuged at 21,000×*g* (4°C) for 20 min. The pellet was suspended in ice-cold 750 mM sucrose 30 mM Tris/HCl pH 8.0 (4°C). Spheroplasts were formed as described by Santini et al. (2000), adding 2 ml of 2.5 mg/ml lysozyme.

pH and temperature study

The activity dependence on pH was determined by the standard assay mixture in which 10 mM Tris/Maleate was used from pH 6.0 to 7.6, 10 mM Tris/HCl from 7.5 to 8.8, 10 mM Glycine/NaOH from 8.6 to 11.0 and 10 mM CAPS/NaOH from 11.0 to 12.0.

The SO activity dependence on temperature was established incubating the enzyme (0.300 μg) in 10 mM Tris/HCl pH 8.0 and at temperatures varying between 25 and 90°C. Thermostability measures were performed incubating the enzyme at 50, 60, 70 and 80°C and determining the SO activity at several times (up to 7 h and 30 min.).

Molecular weight determination

Molecular weight was determined on SDS-PAGE and by analytical gel filtration. SDS-PAGE was performed according to the method of Laemmli (1970), gels were stained for protein using Coomassie Brillant Blue. Standard protein markers (Phosphorylase b, 97 kDa; Albumin, 66 kDa; Ovalbumin, 45 kDa; Carbonic

anhydrase, 30 kDa; Trypsin inhibitor, 20.1 kDa; α-Lactalbumin, 14.4 kDa) were from Amersham. Size exclusion chromatography was performed using the analytical Superdex 75 HR 10/30 (Amersham Pharmacia Biotech), equilibrated in 10 mM Tris/HCl, 150 mM NaCl pH 8.0, and 100 µl of purified SO was separated at a flow rate of 0.5 ml/min. The molecular size was determined using a calibration curve obtained from the retention volumes of standard proteins (Albumin, 67 kDa; Ovalbumin, 43 kDa; Chimotrypsinogen, 25 kDa; Ribonuclease A, 13.7 kDa).

Isoelectric point determination

Isoelectric point was determined using Readystrip IPG strip, 17 cm, pH 3–10 (Biorad) on Protean IEF System (Biorad). The standard proteins were from Biorad. The isoelectrophoresis was performed according to the manufacturer's instructions. Proteins were stained with IEF stain (Biorad).

UV-visible and atomic absorption

The UV-visible absorption of the protein (1.0 mg/ml) was measured using a Cary 100 double-beam spectrophotometer (Varian); analysis of the reduced Tf-SO was performed after extensive deaeration of protein samples and buffers, as well as addition of 400 µM sulfite, immediately before the spectra were recorded.

Molybdenum contents was determined in triplicate and on different enzyme preparations by atomic absorption spectrometry using a Perkin-Elmer apparatus model 5100 equipped with Zeeman graphite furnace and autosampler. The measure was performed according to the manufacturer's instructions.

Isolation of chromosomal *Thermus thermophilus* AT 62 DNA

Chromosomal DNA was extracted by a cesium chloride purification as described by Sambrock et al. (1989). Ethidium bromide and cesium chloride were removed, respectively, by repeated extraction with isoamylic alcohol and extensive dialysis against 10 mM Tris/HCl pH 8.0, 1 mM EDTA. DNA concentration was determined spectrophotometrically at 260 nm, and molecular weight was checked by electrophoresis on 0.8% agarose gel in 90 mM Tris borate pH 8.0, 20 mM EDTA (TBE buffer) using DNA molecular size markers.

Amplification and cloning of SO gene

The gene encoding sulfite oxidase (*SO2*) was amplified via PCR; the 5′ primer used in the amplification contained the NcoI site (underlined in the sequence): SOFw (5′-AGACCATGGACAAGGTAAACCGGCGC-3′). The 3′ primer had the following sequence: SORev (5′-GGATCCCACCACCTTCACCTTGAC-3′), and was designed to insert a recognition site for the BamHI endonuclease (underlined) and to delete the termination codon. The reaction was performed for 35 cycles in a PCR Sprint, Temperature cycling system (Hybaid) under the condition described by Saiki (1990) using as enzyme the Taq polymerase and 55°C annealing temperature. The resulting DNA fragment was ligated into pGEM® - T Easy plasmid previously made end-compatible with the same restriction endonucleases and DNA fragments from 4 independent clones were sequenced by PRIMM DNA Sequencing Service (Naples, Italy). The clone containing the whole exact gene was digested with NcoI and used as template for PfU polymerase. The 5′ primer was SOFw and the 3′ primer was designed to generate the BglII site (underlined): SO2Rev (5′-CCATAGTGATTAGATCTCACCACCT CC-3′). The amplified fragment was digested with NcoI and BglII and inserted in pQE-60 to have a His-tag at the C-terminal: the resulting clone was named pQE-SO2.

The gene lacking the putative peptide leader (*SO2P*) was also amplified via PCR using as template pQE-SO2 digested with NcoI and as primers the following oligonucleotide: SOPFw (5′-TATGTCGACCGAAGGAGG TTTTTC-3′) containing the NcoI site and SO2Rev. PfU polymerase was used as enzyme and the amplified fragment was digested NcoI / BglII and cloned in pQE-60 to have a His-tag at the C-terminal. This construct was named pQE-SO2P.

Expression and purification of the recombinant protein

The *E. coli* TP1000 strain (genotype *ΔmobAB*), previously described as the most advantageous strain for the accumulation of MoCo (Temple et al. 2000), was used for the protein expression. *E. coli* TP1000-competent cells were transformed with pQE-SO2 and pQE-SO2P expression vectors and grown at 37°C overnight in Luria-Bertani medium supplemented with 100 µg/ml ampicillin and 25 µg/ml kanamycin. These cultures were diluted 1:10 into LB medium supplemented with antibiotics and 1 mM Na_2MoO_4 and grown aerobically at 22°C until $OD_{600} = 0.6$. After the addition of Isopropyl-β-D-thiogalactopyranoside (IPTG) to 1 mM final concentration the growth was extended for 6 h and the cells were harvested by centrifugation at 3,000×*g*, at 4°C.

The cell pellets were homogenized 50 mM Sodium phosphate, 300 mM NaCl pH 8.0, and lysed by three cycles of French pressure. After the centrifugation at 65,000×*g*, imidazole was added at the final concentration of 10 mM. The supernatant was loaded into His-select HF Nickel affinity gel (Sigma) and the unbounded proteins washed by 3 column volumes of 50 mM sodium phosphate, 300 mM NaCl, 30 mM Imidazole pH 8.0; the His-tagged protein was eluted with 120 mM Imidazole in 50 mM sodium phosphate, 300 mM NaCl pH

8.0. Fractions with sulfite oxidase activity were pooled and dialyzed against 10 mM Tris/HCl pH 8.0. Purified proteins, named respectively SO2 and Tf-SO2, were checked by SDS-PAGE on 10% polyacrylamide gels.

Results

Production, purification and electrophoretic analysis of SO

In order to induce the sulfite oxidase activity in *T. thermophilus* AT 62 the growth medium A was supplemented with sodium molybdate at increasing concentrations. Molybdenum slightly affected the growth rate of cells (Fig. 1a) but was able to increase the amount of sulfite oxidase activity in the cells (Fig. 1b). In fact, the addition of molybdenum to culture media at a concentration of 2.07 mM molybdate, showed a fourfold increase in the sulfite oxidase activity.

Higher molybdenum concentrations did not show any further increase of the cell growth rate and enzyme activity (data not shown).

We used the optimized growth conditions to scale-up *T. thermophilus* AT 62 cell cultures to amounts suitable for the purification and the characterization of the sulfite oxidase.

Sulfite oxidase from *T. thermophilus* AT 62 (Tf-SO) was purified by four chromatographic steps as summarized in Table 1 for a typical 100-l fermentor scale culture.

The value of the enzyme activity obtained in the crude extract could be considered only apparent and was not indicated in the purification table; at this stage severe interference of proteins or other components of the crude extract with a redox potential very similar to ferricyanide occurred in the assay, as already reported by Cohen and Fridovich (1971a, b) for the purification of the enzyme from bovine liver. The enzyme activity measured in the active fractions pooled from DEAE-52 was used as initial value in the calculation of purification factor and yield. The crucial purification step was the final Mono Q chromatography resulted in the elution of an homogeneous protein but with a yield of only 14% of SO activity.

The purified protein shows a single coomassie stained band at ~39.1 kDa (Fig. 2a) on SDS-PAGE and an isoelectric point of 6.8 on isoelectrofocusing gel. The molecular mass determined by SDS-PAGE is in agreement with the value of ~ 38 kDa obtained after gel filtration on analytical Superdex 75, identifying the enzyme as a monomer. The sulfite dependent activity of this protein was demonstrated by enzymatic assay on native PAGE (Fig. 2b) identifying definitively the purified protein as a sulfite oxidase.

Subcellular localization of SO from *T. thermophilus* AT 62

The cellular localization of Tf-SO, was identified by fractionation of *T. thermophilus* AT 62 cell compartments and analysis of the periplasmic fraction, prepared according to the protocol of Santini et al. (2000). The extent of spheroplast formation was estimated by observing the suspension under a phase contrast microscope: spheroplasts appear as small spheres and intact cells as thin rods. Both fractions, periplasm and cytosol from spheroplasts, were tested for sulfite oxidase activity, revealing that the enzyme was mainly located in the periplasm compartment, with 18% of total activity detectable in the cytosolic fraction; this value could nevertheless be ascribed to intact cells, persistent and distinguishable at the microscope analysis, that were colleted together with spheroplasts after the mild lysis treatment.

Spectroscopic analysis of Tf-SO

The presence of molybdopterin in the enzyme structure was ascertained by performing UV–visible absorption spectra of purified Tf-SO samples. The analysis (Fig. 3) also revealed the absence of the characteristic peaks

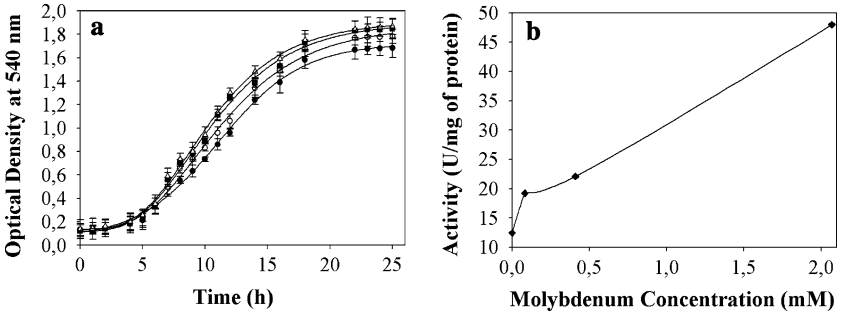

Fig. 1 Dependence of molybdenum concentration on *T. thermophilus* AT62 growth (**a**) and SO activity (**b**). **a** Growth of *T. thermophilus* AT62 cells in medium A (glucose–tryptone–yeast extract) (*filled circle*), in medium A supplemented with 0.083 mM (*open circle*), 0.41 mM (*filled square*) and 2.07 mM (*open triangle*) sodium molybdate. **b** Sulfite oxidase activity (U/mg of protein) in *T. thermophilus* AT62 at the different molybdenum concentrations

Table 1 Purification of sulfite oxidase from 40 g of frozen *T. thermophilus* AT62 cell material

	Volume (ml)	Activity (U)	Protein content (mg)	Specific activity (U/mg)	Purification factor (fold)	Yield (%)
DEAE 52	40	32.16	168.1	0.191	0	100
YM10	3	30.9	162.5	0.190	0.99	96
S-75	13	27.6	19.1	1.44	7.54	89
HTP	35	24.3	4.1	5.93	31	88
Mono Q	0.25	3.4	0.06	56.67	297	14

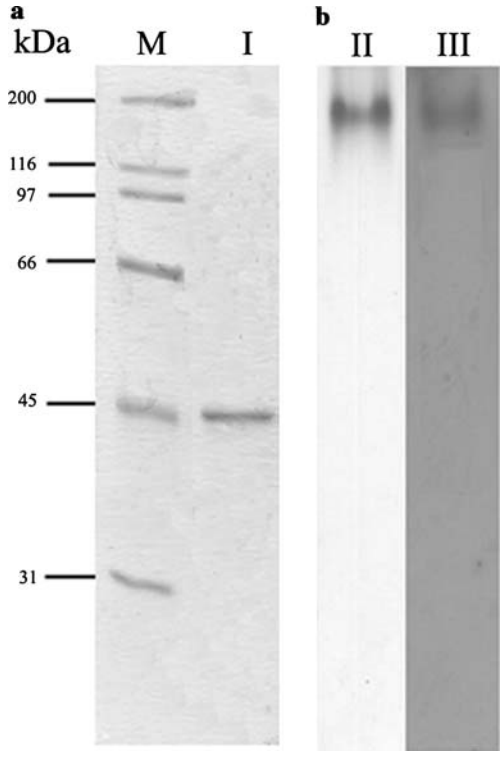

Fig. 2 10% SDS-PAGE (**a**) and 7.5% native PAGE (**b**) of purified Tf-SO. The analysis were performed on protein after all steps of purification. Lane M: molecular weight markers; *Lane I* purified SO from *T. thermophilus* AT62; *Lane II* coomassie staining of purified Tf-SO; *Lane III* activity staining of Tf-SO as described in Materials and methods

corresponding to prosthetic heme groups. The absorption maximum at ~350 nm with a broad shoulder between 400 and 500 nm (upper line in Fig. 3) can be assigned to the electronic transitions molybdenum (VI)–cysteine and enedithiolate-to-molybdenum charge-transfer bands (Garton et al. 1997), respectively. In fact they are lost in the partially reduced enzyme (lower band in Fig. 3), as in case of *A. thaliana* SO (Eilers et al. 2001), of tryptically cleaved rat SO (Johnson and Rajagopalan 1977) and of the recombinant MoCo domain of human SO (Garrett and Rajagopalan 1996).

Furthermore, to confirm the presence of molybdenum in the protein structure we performed an atomic absorption spectroscopy analysis. 3.83 pmol molybdenum could be measured relatively to 3.91 pmol of the SO used for the analysis. Therefore, like in other members of sulfite oxidase family (Kisker 2001; Johnson and Rajagopalan 1977), the molybdenum is present in a ratio of 1 atom for molecule of protein.

Kinetics properties

Tf-SO exhibited no sulfite oxidase activity when cytochrome *c* was used as electron acceptor showing a sulfite dependent activity only when ferricyanide was used as substrate.

The kinetic parameters were determined for both substrates: sulfite and ferricyanide. The K_M value for sulfite was 10.67 ± 0.51 μM, when calculated by Lineweaver-Burk plot, according to the value determined by Michaelis-Menten representation (11.0 ± 0.8 μM) (Fig. 4a). This value is very similar to the constant determined for human (Cohen and Fridovich 1971a, b) and chicken (Kisker 2001) SO (17 and 19.1 μM, respectively) and lower respect to *Arabidopsis* (Eilers et al. 2001) and *Thiobacillus* SO (Kappler et al. 2000) (33.8 and 33 μM, respectively). The K_M value for ferricyanide was 6.33 ± 0.68 μM (Fig. 4b). The v_{max} and K_{cat} values for sulfite and ferricyanide were determined by extrapolation from Lineweaver-Burk plots and were respectively 11.7 ± 1.0 μM min^{-1} and $53{,}318 \pm 832$ s^{-1}. The K_{cat}/K_M ratio was 4,983 μM^{-1} s^{-1} for sulfite and 8,009 μM^{-1} s^{-1} for ferricyanide.

The enzymatic activity was tested in presence of increasing ionic strength values: SO activity increased at low salt concentrations, while higher concentrations were inhibitory. The inhibitions rate was also depending on the nature of monovalent ion.

In particular the presence of potassium ion in the range of 45–100 mM produced the maximum effect of activation, increasing of 188% the Tf-SO activity; 45 mM of Na^+ was enough to increase the SO activity to 154% while the NH_4^+ produced the maximum of activation (174%) at a concentration of 30 mM. On the basis of these results, unlike hepatic (Kessler and Rajagopalan 1974), *Arabidopsis* (Eilers et al. 2001) and *Thiobacillus* (Kappler et al. 2000) SOs found to be inhibited by increasing ionic strength already at very low values, Tf-SO was inhibited at much higher (~400 mM) salt concentrations.

All measures were performed in the presence of non-interfering concentration of Tris buffer (10 mM). In these conditions different concentration of phosphate

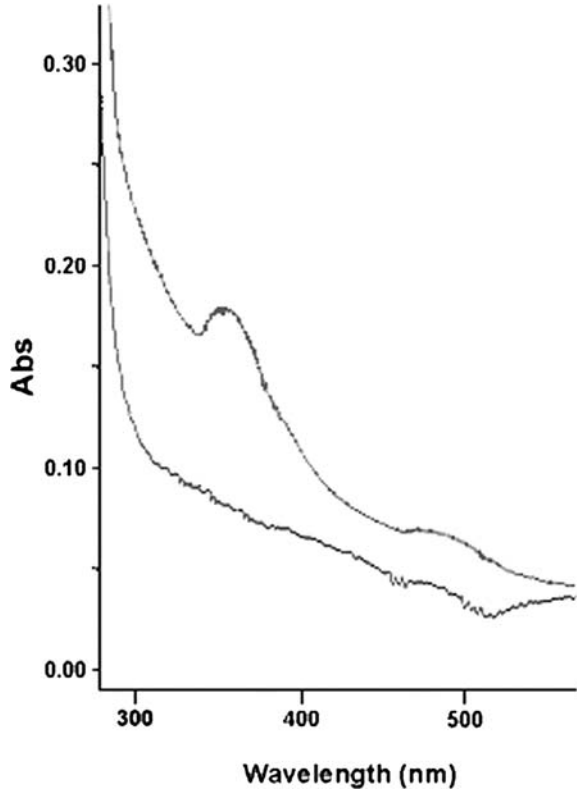

Fig. 3 Tf-SO UV-Visible spectrum. UV-visible spectra of oxidized and partially reduced Tf-SO. Absorption spectra of oxidized (lower line) and sulfite-reduced (upper line) enzymes (1.0 mg/ml) were recorded in 10 mM Tris/HCl (pH 8.0). The 360- and 480-nm absorption bands of the oxidized enzyme are due to cysteine-to-molybdenum and enedithiolate-to-molybdenum charge transfer bands, respectively

buffer (10–200 mM) showed no effect on Tf-SO activity, differing from the SO from eukaryotic and several prokaryotic sources (Cohen and Fridovich 1971a, b; Eilers et al. 2001; Kappler et al. 2000; Kessler and Rajagopalan 1974).

Increasing nitrate concentrations (up to 7 mM) did not affect the enzymatic activity, whereas concentration of only 3 mM produce a 50% inhibition in the case of *Thiobacillus* SO (Kappler et al. 2000).

Similarly, Tf-SO did not exhibit product inhibition by sulfate up to 80 mM, a concentration that completely inhibits *Thiobacillus* (Kappler et al. 2000) and chicken (Kessler and Rajagopalan 1974) SOs.

The Tf-SO enzyme was inactivated by Ca^{2+} ions with a K_i value of 9.4 mM as calculated by Dixon plot. This result was also confirmed by the activation effect of EDTA on Tf-SO activity: the EDTA could chelate any Ca^{2+} ions eventually present in the solution.

Arsenite and periodate are also known as sulfite oxidase inactivators (Gardlik and Rajagopalan 1991) and studied in more details: the inactivation by arsenite is the result of arsenite replacement of Mo during the redox reaction of arsenite to arsenate, while the periodate directly attacks the bond between Mo and the protein. Sulfite oxidase from *T. thermophilus* AT62 is inactivated by arsenite (50% of inactivation at 7.4 mM) but not by periodate.

Enzyme characterization

The pH dependence of the reaction catalyzed by SO from *T. thermophilus* AT62 was determined in several buffer: the results are showed in Fig. 5.

The maximal value of activity was found at pH 11.0, higher than that of other homologue enzymes (Cohen and Fridovich 1971a, b; Kessler and Rajagopalan 1972; Eilers et al. 2001; Kappler et al. 2000), but this activity increase could be only apparent. In fact, high temperature, necessary for the optimal enzyme activity in vivo (presumably close to the optimum temperature for *Thermus* growth) considerably increases the non-enzymatic oxidation of sulfite. The increasing pH produces a similar effect favoring this chemical reaction. For this reason the limit temperature of 60°C and pH 8.0 were the conditions chosen to exclude these side effects in the enzymatic analysis of SO from *T. thermophilus* AT62.

SO thermostability and thermophily were also determined, revealing that Tf-SO is a very heat stable

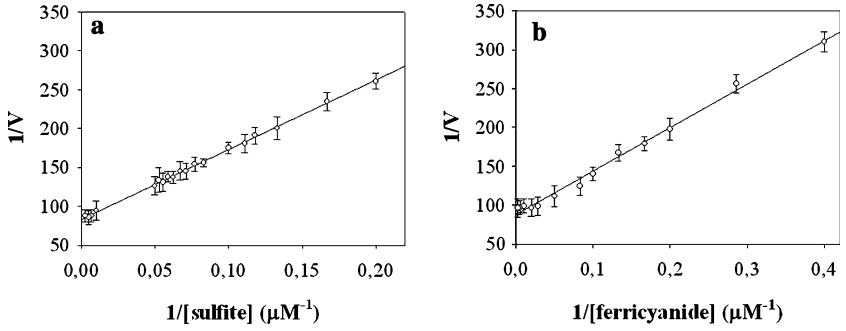

Fig. 4 Kinetic parameters of Tf-SO. Lineweaver – Burk plot of steady state kinetic of SO for sulfite (**a**) and for ferricyanide (**b**). The reaction was initiated with 0.300 μg of purified Tf-SO. Steady-state dependence of catalysis on sulfite was conducted using constant 400 μM ferricyanide and varying the sulfite concentration between 5 and 400 μM. The study of ferricyanide concentration was conducted using constant 400 μM sulfite and varying the ferricyanide concentration between 5 and 400 μM

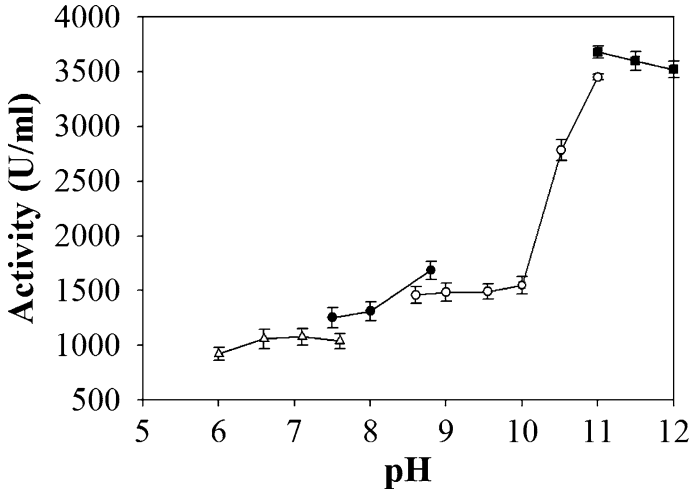

Fig. 5 pH dependence on Tf-SO activity. The assay was performed in a double beam spectrophotometer at the pH values indicated and at 60°C. The buffers used were: Tris/Maleate (pH 6.0–7.6) (*open triangle*), Tris/HCl (pH 7.5–8.8) (*filled circle*), Glycine/NaOH (pH 8.6–11.0) (*open circle*), CAPS/NaOH (pH 11.0–12.0) (*filled square*). All measures were taken in triplicate using as reference sample a mix containing all the varying components of the assay and no enzyme

enzyme retaining about 100% of residual activity up to 7 h of incubation at 50°C. Half life time of the enzyme (Fig. 6a) at 60, 70 and 80°C, are respectively 8, 6.5, and 1.5 h. The optimum temperature of the enzyme activity was 60°C (Fig. 6b), but the enzyme retain the 30% of its activity at 25°C and the 45% at 90°C.

Cloning SO gene

Two possible ORFs encoding putative sulfite oxidases were found by the analysis of the genome sequence of *Thermus thermophilus HB27* (Henne et al. 2004) a strain philogenetically very close to the AT62, used in this study: TTC1046 (named *SO1*) annotated as sulfite dehydrogenase *sox*C precursor, and TTC0961 (named *SO2*) indicated as a probable sulfite reductase. The BLASTP (Altschul et al. 1997) and FASTA (Pearson 1990) alignments of both sequences showed significant similarity with the other sulfite oxidases from eukaryotes and prokaryotes. In Fig. 7 is reported the multiple-sequence alignment with hierarchical clustering, available at the website http://prodes.toulouse.inra.fr/multalin/multalin.html, of the protein sequence translated from the TTC0961 ORF with the orthologs that have been characterized biochemically. In particular, the alignment with the SOs from chicken liver, *T. novellus* and the plant *A. thaliana* produced identity scores of 30, 35 and 27%, respectively.

Moreover, the search for structural motifs involved in known biochemical functions, performed with the PFAM (Protein Families database of Alignments and HMMs) program available at the website http://www.sanger.ac.uk, revealed a general catalytic oxidoreductase molybdopterin binding domain (residues 44 to 365) and a MoCo oxidoreductase dimerization domain (residues 283 to 361) that are present in all of the sulfite oxidases compared. Interestingly, as in the plant enzyme, no heme binding domain common to animal and *T. novellus* SOs was found in the N-terminal region of the protein.

The molecular mass and isoelectric point prediction, performed with a tool available on the website http://www.expasy.org on both protein sequences directed our attention on *SO2* for further analysis, the mature polypeptide showing a molecular mass and an isoelectric

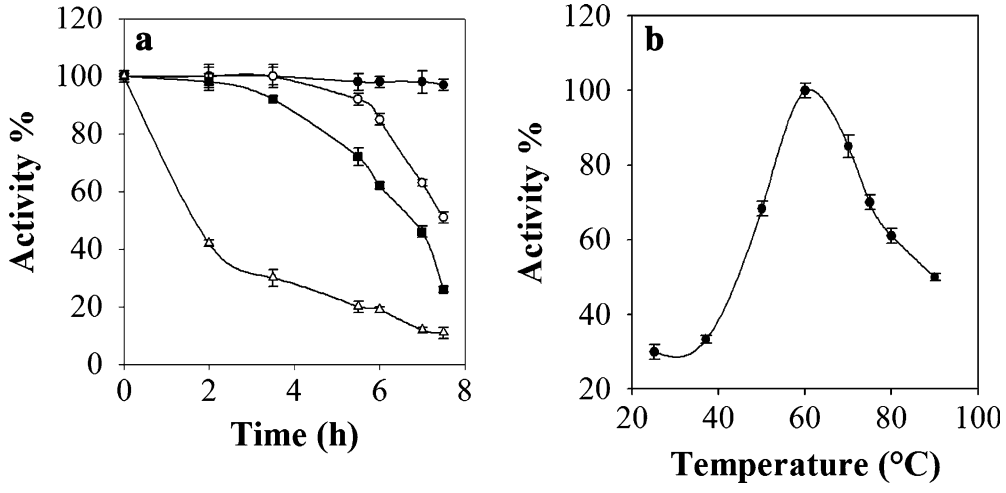

Fig. 6 Thermostability (**a**) and thermophilicity (**b**) of Tf-SO. Thermoresistance of the *T. thermophilus* AT62 sulfite oxidase was investigated in the temperature range of 50–80°C and after the different incubation times indicated and performing the assay of residual activity at 60°C [(*filled circle*) 50°C, (*circle*) 60°C, (*filled square*) 70°C and (*open triangle*) 80°C]. The optimum temperature was determined by the ferricyanide assay in the range 25–90°C of temperature and using 0.300 μg of Tf-SO

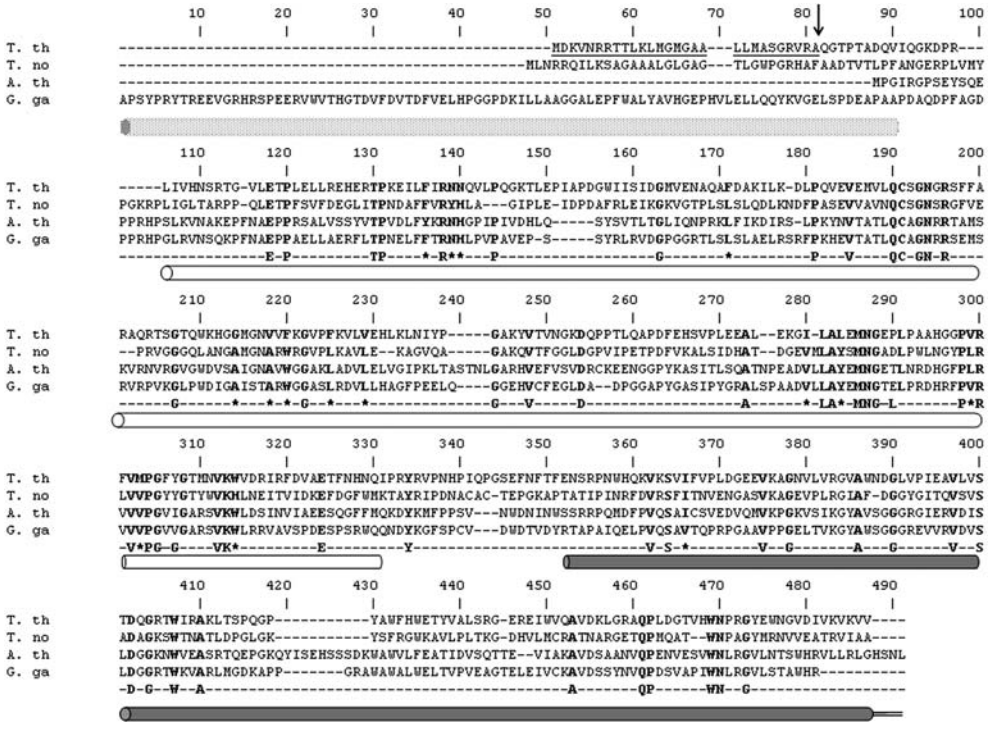

Fig. 7 Multiple alignment of the *Thermus thermophilus HB27* sulfite oxidase sequence with the sequences of SOs from different sources. The protein sequence translated from the TTC0961 open reading frame was compared to both eukaryotic and bacterial counterparts that have been biochemically characterized, and significant similarity was found with the sequences indicated. *T. th*, *T. thermophilus* HB27 SO; *T. no*, *T. novellus* SO; *A. th*, *A. thaliana* SO; *G. ga*, chicken liver SO. The search for structural motifs involved in known biochemical functions revealed the presence of a conserved central region, namely, an oxidoreductase molybdopterin binding domain (*open bar*) and a C-terminal MoCo oxidoreductase dimerization domain (*dark gray bar*) and the absence of the N-terminal heme binding domain (*light gray bar*). The predicted peptide leader on *T. thermophilus* AT62 sequence (*underlined*) and the hypothetical proteolytic cleavage site (marked by *arrow*) are also shown

point in more significant agreement with the values determined for wild type Tf-SO.

The *SO2* gene sequence was analyzed for subcellular sorting and signal peptide cleavage sites, with PSORT (Gardy et al. 2005) and SignalP 3.0 (Bendtsen et al. 2004) programs, available on the same website. The prediction analysis indicated a periplasmic location and an hypothetical proteolytic cleavage site with 99% of probability between residues 28 and 29. By PCR amplification on *T. thermophilus* AT62 genomic DNA and using oligonucleotides derived from *SO2* sequence as primers we amplified the sulfite oxidase gene. Sequence analysis on all clones selected showed that SO gene from *T. thermophilus* AT62 has two nucleotide substitution when compared to the strain HB27 SO gene producing the amino acid replacement Glu100 with Asp and Ala297 with Val. *SO2* gene was cloned and expressed in TP1000 *E. coli* cells (*ΔmobAB*) as a C-terminal His-tag protein both as the entire ORF and a derivative, named *SO2P*, lacking the nucleotides encoding the peptide leader. The intact *SO2* expression produced a protein which mostly accumulated in the inclusion bodies; excision of the leader peptide allowed complete solubilization of the enzyme during expression. In fact, *SO2P* was expressed as an active enzyme, indicating that correct processing to mature protein of the precursor could not occur in *E. coli*. Therefore the *SO2P* gene product (Tf-SO2) could be isolated and characterized: the protein was purified to more than 98% by a single step purification by affinity chromatography on a nickel chelating gel.

Characterization of the recombinant enzyme

The *SO2* gene mature products expressed both in *T. thermophilus* and *E. coli* did not show relevant differences both at the kinetic and structural levels. The optimum pH of recombinant enzyme was 10.0, slightly different from the value obtained for the native protein. Likewise, the highest activity was achieved at 60°C, showing however a faster decrease with the rise of the temperature (data not shown). The enzyme was very thermostable retaining 64% of his activity after 6.5 h at 60°C. The enzymatic activity was tested also at regular time intervals over 38 days: after incubation of 1 month at room temperature 70% Tf-SO2 activity was recovered (Fig. 8). The kinetics parameters were also determined showing a little increase of the K_M value (52.6 μM). The recombinant and native enzymes showed similar electrophoretic mobility both under native and denaturing conditions with slight differences due to the presence of the his-tag sequence on the *E. coli* product.

Discussion

A screening for whole cell sulfite oxidase activity of different thermophilic microorganisms revealed that *Thermus* species are very active in the expression of high catalytically efficient enzymes. In particular, the gram-negative *T. thermophilus* AT62 was chosen because it showed the highest total activity. In fact, under conditions of specific enzyme induction upon molybdenum exposure which raised up to fourfold the protein expression, the strain provided enough material for successful purification in a few steps and further study.

Molybdenum sulfite oxidase induction in bacteria is not unusual. A similar result was also reported by Southerland and Toghrol (1983) that noted a 10-fold increase of the sulfite oxidase activity in *T. novellus* upon addition of 4.0 μM sodium molybdate in the autotrophic growth medium.

The protein from *T. thermophilus* AT62 described here is clearly a member of the molybdenum containing sulfite oxidase family but the kinetic analysis, performed following the oxidation of sulfite and with ferricyanide as the electron acceptor, already pointed out major differences with representatives from both bacterial and eukaryal sources. Johnson and Rajagopalan (1977) have demonstrated that in rat hepatic SO, the electron transfer between the molybdenum and cytochrome *c*, the physiological electron acceptor, is mediated by the heme prosthetic group. The *Thermus* enzyme displayed direct sulfite oxidation with no need for cytochrome-like proteins as intermediate in the electron transfer thus suggesting a strong similarity with the enzyme isolated from peroxisomes of the plant *A. thaliana* (Eilers et al. 2001). The resemblance was confirmed at the structural level with a similar lower molecular mass (about 40 kDa) when compared to the well characterized bird and human enzymes and the absence of a polypeptide bound heme group undetectable as a characteristic peak in UV/visible spectra. Nor a cytochrome-like polypeptide was found associated to the enzyme during all steps of purification, including gel exclusion chromatography, and/or after prolonged incubation of the pure enzyme with both cell free and periplasmic protein extracts.

The enzyme was endowed with the highest thermostability described for a sulfite oxidase and high catalytic efficiency at the optimum temperature and unusually high pH values, with substrate affinity among the lowest so far determined.

The low cellular amount of the protein posed a limit to its full structural and functional characterization; moreover, both investigation about the physiological role and evolutionary relationship of the enzyme required the identification and cloning of the gene and the characterization of the gene product in an overexpressing heterologous system. Therefore, a search aimed at the identification of SO genes was performed on the sequence of the closely phylogenetically related *T. thermophilus* HB27 genome recently completed and made available. Nevertheless, two different putative ORFs encoding SOs, both lacking a heme binding domain, were recognized and the mere sequence analysis did not help in the assignment of the specific gene to the enzyme characterized in vivo. Therefore, the identification and the characterization of the gene products in *E. coli* was necessary also for this reason. One sequence was strictly related to *sox*C genes found in other prokaryotes as associated in operons with other membrane cytochrome and flavoprotein genes to cooperate in the sulfite metabolism (Southerland and Toghrol 1983). Once cloned in *E. coli* the encoded protein from this soxC-like gene did not show the same features of Tf-SO and among other differences, it had no ferricyanide-dependent activity (data not shown). The other ORF (named *SO2*) appeared to be the best candidate for the features predictable from the translated gene product (pI, molecular mass etc.) more significantly similar to those determined for the wild type Tf-SO protein. Interestingly, under the growth conditions tested, *T. thermophilus* cell extracts showed the single prominent enzyme

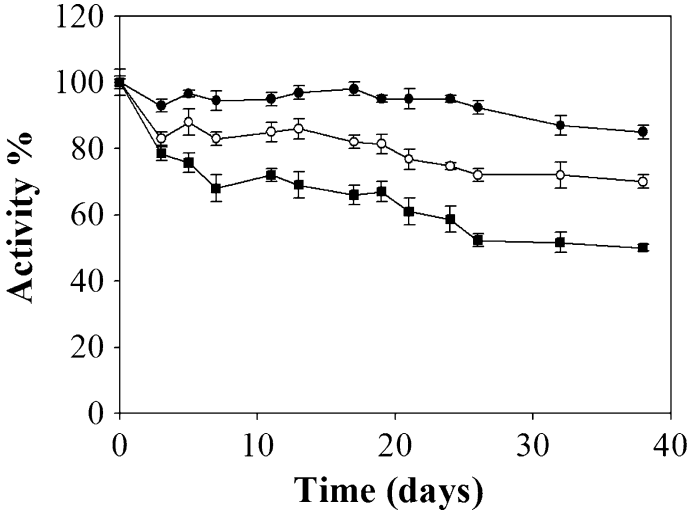

Fig. 8 Stability of recombinant Tf-SO2. The enzymatic activity was assayed for 38 days after incubation at three different temperatures (4°C (*filled circle*), 25°C (*circle*) and 45°C (*filled square*)) as described in Materials and methods

characterized in this study. For this reason we assessed a high level expression system of the *SO2* gene cloned in *E. coli* by optimization of both the growth time of the transformed cells and the induction time with IPTG. The expression system and the purification procedure described allowed us to obtain fully active protein in a very large amount: 1,000-fold activity over the wild type protein amount produced by *T. thermophilus*. The crystallization of the protein is currently being carried out with the purpose of obtaining information about its three-dimensional structure.

Very different localizations are reported for sulfite oxidases. In higher eukaryotes the enzyme is present in the mitochondrial intermembrane space (Kisker 2001); as already mentioned in *A. thaliana* in peroxisomal fraction (Eilers et al. 2001) and in most prokaryotes, such as *T. novellus* (Kappler et al. 2000; Kappler and Dahl 2001), it is located in the periplasmic space.

Similarly, wild type SO in *T. thermophilus* was located in the periplasm; the identified *SO2* gene showed indeed a 5′ leader sequence encoding a signal peptide typical for sorting into periplasm in gram-negative bacteria and could be expressed in *E. coli* only when this short sequence was deleted. Although, as already mentioned, a specific electron acceptor could not be identified by association as the physiological partner in vivo, the analysis of the *SO2* genomic environment pointed out the presence of a cytochrome-like ORF (TTC0962) starting at 69 bp downstream of the *SO2* gene 3′ end. Whether this gene organization is only haphazard or whether ORF TTC0962 encodes the real electron acceptor of Tf-SO catalyzed sulfite reduction in *T. thermophilus* is currently under investigation.

Why *T. thermophilus* possesses two different genes displaying sulfite oxidase activity in the periplasmic compartment remains unclear. One can argue that the product of *sox*C gene is membrane bound and somehow linked to the respiratory chain via electron transfer from thiosulfate mediated by the entire SO gene cluster. On the other hand Tf-SO2 is a soluble protein freely diffusing in the periplasmic space and hence able to scavenge environmental or thiosulfate generated sulfite. The assignment of the specific functional role of the sulfite oxidase/dehydrogenase sequences found on the *T. thermophilus* genome and the involvement in the specific sulfite metabolic pathways, is currently under investigation.

To the best of our knowledge the sulfite oxidase from *T. thermophilus* is the first thermostable bacterial enzyme lacking a heme binding domain in a covalently linked form and/or as an associated independent subunit. Its structural and kinetic properties that are more similar to plant counterparts as well as its noticeable resistance to heating show the general feasibility of this enzyme as a study model to facilitate further research both at the genetic and evolutionary exploitation levels.

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