Purification and properties of a thermostable fumarate hydratase from the archaeobacterium *Sulfolobus solfataricus*

SUSANNE PUCHEGGER, BERNHARD REDL* and GEORG STÖFFLER

Institut für Mikrobiologie (Medizinische Fakultät), Universität Innsbruck, Fritz Pregl Str. 3, A-6020 Innsbruck, Austria

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Fumarate hydratase (EC 4.2.1.2) from the extremely thermophilic archaeobacterium *Sulfolobus solfataricus* has been purified to homogeneity by a rapid purification procedure using affinity chromatography and high-performance size-exclusion chromatography, and the enzyme's physical and biochemical properties have been determined. The native enzyme has a molecular mass of 170 kDa and is composed of identical subunits with a molecular mass of 45 kDa, thus indicating a tetrameric structure similar to fumarases isolated from other organisms. The enzyme was active at temperatures ranging from 40 °C to 90 °C, with a maximum activity at 85 °C. The pH optimum for generation of fumarate was found to be pH 8.0. The enzyme showed high stability to denaturation by heat and organic solvents.

Introduction

Fumarate hydratase, or fumarase (EC 4.2.1.2), which catalyses the reversible hydration of fumaric acid to L-malate, is an integral part of the tricarboxylic acid (TCA) cycle. It is widely distributed in plants, animals and bacteria. Most studies have been concerned with mammalian fumarases, which function in the mitochondrial TCA cycle and in cytosolic metabolism (Kanarek *et al.*, 1964; Kobayashi *et al.*, 1981; Kobayashi & Tuboi, 1983; Sacchettini *et al.*, 1986; Beeckmans & Kanarek, 1982).

Fumarases from micro-organisms have been studied less extensively. In *Escherichia coli* it was found that there were two biochemically distinct classes of fumarases, which may function not only in the TCA cycle, during aerobic metabolism, but also, under anaerobic conditions, in the ultimate reduction of malate by providing fumarate as an anaerobic electron acceptor (Woods *et al.*, 1988). One of these fumarases (class II) exhibits remarkable sequence homologies and biochemical properties with fumarases of *Bacillus subtilis*, *Saccharomyces cerevisiae* and human liver (Woods *et al.*, 1986; Wu & Tzagoloff, 1987).

Since archaeobacteria represent a third line of evolution, alongside eubacteria and eukaryotes (Woese & Fox, 1977), the study of enzymes from these organisms may increase our understanding of protein evolution.

Furthermore, some enzymes from extremely thermophilic archaeobacteria have not only a high thermostability, but also a high chemostability (Buonocore *et al.*, 1980; Giardina *et al.*, 1986) and may therefore have biotechnological applications.

In this paper, we describe the purification and characterization of the thermostable fumarase of *Sulfolobus solfataricus*, an extremely thermophilic sulphur-dependent archaeobacterium, which was isolated from hot acid habitats.

Methods

Organism and culture conditions. *S. solfataricus*, strain DSM 1616, was originally isolated from a volcanic hot spring (Brock *et al.*, 1972). Cells were grown at 85 °C in a 4 l glass fermenter with low agitation, and aeration flux of 30 ml min⁻¹ (l of broth)⁻¹. The culture medium contained: 3.1 g KH₂PO₄, 2.5 g (NH₄)₂SO₄, 0.2 g MgSO₄·7H₂O, 0.25 g CaCl₂·2H₂O, 2.0 g yeast extract in 1 l of distilled water. The pH of the medium was adjusted to 3.5 with 10 M-H₂SO₄. Cell growth was monitored by following the optical density at 540 nm. At an OD₅₄₀ of 0.6 the pH of the medium was adjusted to 6.0 by adding 5 M-NaOH dropwise. Cells were then put on ice and harvested by centrifugation (15 min at 10000 g).

Enzyme assay. Fumarase was routinely assayed at 70 °C in 100 mM-potassium phosphate buffer (pH 7.5) containing 50 mM-L-malate, by monitoring the production of fumarate as a change in absorbance at 240 nm. The measurements were made over a period of 10 min in a Beckman spectrophotometer equipped with a thermostatically controlled autosampler. One unit of enzyme activity (U) is defined as the amount of enzyme catalysing the formation of 1 µmol of fumarate per

Abbreviation: TCA, tricarboxylic acid.

min, using an extinction coefficient for fumarate of $2.44 \text{ mM}^{-1} \text{ cm}^{-1}$ at 240 nm.

Purification of fumarase. All purification steps were carried out at 4°C .

(i) **Crude extract.** Wet-frozen cells (7 g) were thawed in 14 ml of buffer A (50 mM-Tris/HCl buffer, pH 7.5) and disrupted by passing them twice through a French pressure cell operated at a pressure of 97 MPa. The suspension was centrifuged at $40000 g$ for 45 min at 4°C .

(ii) **Precipitation with protamine sulphate.** Nucleic acids were precipitated by adding 0.2 ml 2% (w/v) protamine sulphate in buffer A per ml of crude extract. After 60 min at 4°C , with slow stirring, the precipitated material was removed by centrifugation at $40000 g$ for 30 min at 4°C . The supernatant was dialysed against several changes of buffer B (20 mM-Tris/HCl buffer pH 6.8, 5 mM- MgCl_2 , 0.4 mM-EDTA) overnight.

(iii) **Dye affinity chromatography on Affi-Gel Blue gel.** NAD, NADH, NADP and NADPH were added to the protein solution, to a final concentration of 0.5 mM with respect to each pyridine nucleotide. This solution was applied to an Affi-Gel Blue (Bio-Rad) column ($16 \times 60 \text{ mm}$) previously equilibrated with buffer B. The column was washed with 2 vols (20 ml) of buffer B containing 0.1 mM of each pyridine nucleotide. Affinity elution was performed with a linear gradient (30 ml) from 0 to 10 mM fumarate in buffer B at a flow rate of 40 ml h^{-1} . Fractions containing fumarase activity were pooled and concentrated.

(iv) **High-performance size-exclusion chromatography.** The concentrated protein solution was applied to a Bio-Sil (Bio-Rad) TSK 250 column ($600 \times 7.5 \text{ mm}$) and eluted with 20 mM-phosphate-buffered saline (20 mM-sodium phosphate, 0.15 M-NaCl, pH 7.0), at a flow rate of 1 ml min^{-1} . Fractions showing fumarase activity were pooled and stored at -80°C .

Electrophoresis and isoelectric focusing. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to check protein purity, and to determine the molecular mass of the purified enzyme under denaturing conditions. Protein samples were boiled for 5 min in 10 mM-sodium phosphate, pH 7.0, 2% (w/v) SDS and 1% (v/v) 2-mercaptoethanol, prior to electrophoresis on 10% (w/v) polyacrylamide slab gels using a Mini-Protean electrophoresis unit (Bio-Rad). After electrophoresis, protein bands were visualized by a silver staining procedure (Merill *et al.*, 1981). The subunit molecular mass was determined using the Sigma MW-SDS-200 calibration kit.

The isoelectric point (pI) of the fumarase was determined with an LKB Multiphor apparatus using broad-range pH (3.5–9.5) thin-layer polyacrylamide gels (Görg *et al.*, 1978). Appropriate Pharmacia calibration kits were used. The proteins were stained with Coomassie Brilliant Blue R and densitometer scans were used to assess the pI.

Molecular mass estimation of native enzyme. The purified fumarase was chromatographed on a Bio-Gel (BioRad) A 1.5 gel filtration column ($8 \times 800 \text{ mm}$), calibrated with ferritin (450 kDa), aldolase (160 kDa), bovine albumin (67 kDa) and chymotrypsinogen A (25 kDa). The eluting buffer was buffer B with NaCl added to 100 mM; fractions of 1 ml were collected at a flow rate of 1.9 ml h^{-1} . A computing integrator was used for monitoring peak area and retention time. The molecular mass of the purified native enzyme was also estimated by sucrose-density-gradient centrifugation as described by Martin & Ames (1961). An enzyme sample (30 μg in 0.2 ml) was layered onto a preformed 10–30% sucrose gradient. Catalase (240 kDa), albumin (67 kDa) and horse myoglobin (17 kDa) were used as markers. Centrifugation was carried out at 4°C and 28500 r.p.m. for 15 h in a Beckman SW41 rotor.

pH and temperature relationships. The enzyme activity as a function of pH was tested using 50 mM-potassium phosphate for pH values 5.5–7.5 and 50 mM Tris/HCl for pH values 7.0–9.0 by the assay described

above. NaCl was added to each buffer to bring the ionic strength to the same value. All pH values were adjusted at the appropriate temperature. The relationship between fumarase activity and temperature was determined at between 30°C and 95°C , using the standard assay.

Kinetics. The kinetic parameters of *S. solfataricus* fumarase for L-malate and fumarate were determined at 70°C , using the activity assay described above, but 50 mM-Tris/acetate, pH 7.0, was used as buffer instead of potassium phosphate. Fumarate was used as substrate instead of L-malate for determination of K_m for fumarate. The disappearance of fumarate was monitored as a decrease in absorbance at 240 nm. The K_m values were estimated by the double reciprocal plot method.

Results and Discussion

Enzyme purification

A rapid two-step procedure using affinity chromatography and high-performance size-exclusion chromatography was used to purify fumarase from *S. solfataricus*.

The crude extract was loaded onto an Affi-Gel Blue column in a buffer containing all four pyridine nucleotides to prevent the binding of pyridine-nucleotide-binding enzymes to the column. When all unbound proteins had passed through the column, fumarase was specifically eluted with a linear gradient of 0–10 mM-fumarate in the absence of pyridine nucleotides. For some preparations, proteins were fractionated by ammonium sulphate (55–70% saturation) prior to loading onto the column. After elution with fumarate, the protein fraction appeared to be almost homogeneous; however, using this purification scheme, the overall yield was significantly lower. In each case an additional 3–5% of the bound fumarase activity was eluted with 0.5 M-NaCl; however, this fraction contained a large amount of contaminating proteins. For final purification, fractions showing fumarase activity were pooled and applied to a size-exclusion HPLC column as described in Methods. The progress of purification was monitored by SDS-PAGE (Fig. 1), and a typical purification is summarized in Table 1. From this it is evident that the most effective purification step was the dye-affinity chromatography.

Molecular mass and isoelectric point

The purified enzyme was detected as a single band with a molecular mass of 45 kDa using SDS-PAGE (Fig. 1, Lane 3).

The molecular mass of the native enzyme was estimated by gel filtration chromatography and by sucrose gradient centrifugation. The values obtained were 170 kDa and 160 kDa respectively, suggesting that the native protein is composed of four identical subunits.

With respect to the subunit molecular mass and the

Table 1. Purification of fumarase from *S. solfataricus*

Step	Total protein (mg)	Total activity (U)	Specific activity (U mg ⁻¹)	Purification yield	
				(-fold)	(%)
Cleared lysate	237.0	192	0.81	1	100
Protamine sulphate	214.0	179	0.84	1	93
Affi-Gel Blue	1.5	65	43.33	53	34
Size-exclusion HPLC	0.2	41	205.00	253	21

Fig. 1. Progress of fumarase purification monitored by SDS-PAGE. Samples from different purification steps were electrophoresed on 10% acrylamide slab gels, and proteins were visualized by silver staining. The lanes contained: 1, 25 µg of crude cell extract; 2, 2.5 µg of proteins eluted from Affi-Gel Blue gel with fumarate; 3, 1.5 µg of purified fumarase; 4, molecular mass markers.

tetrameric structure the *S. solfataricus* fumarase closely resembles fumarases purified from *B. subtilis* and pig heart and a fumarase from *E. coli* encoded by the gene *fumC* (Shibata *et al.*, 1985; Kanarek *et al.*, 1964; Woods *et al.*, 1988). It is different from a fumarase from *Euglena gracilis* and two additional fumarases from *E. coli* (encoded by the genes *fumA* and *fumB*), which are dimeric proteins with subunit molecular masses of 60 kDa (Shibata *et al.*, 1985; Woods *et al.*, 1988).

The pI of the fumarase was estimated to be 7.1 by isoelectric focusing.

Substrate specificities and kinetic studies

The substrate specificities of the enzyme were tested, using D-malate, maleate, citramalate and citrate (obtained from Sigma) instead of L-malate or fumarate, but no activity could be detected with these substrates. This clearly demonstrates that the enzyme is a fumarase, rather than a hydratase with a broad substrate specificity that includes fumarate.

Purified enzyme was used for determination of the kinetic properties and tests were performed in 50 mM-Tris/acetate (pH 7.0). The rate of fumarate or malate

formation was measured over a range of substrate concentrations of 0.1–10 mM-L-malate and 0.025–1 mM-fumarate respectively. The K_m for malate was 0.30 mM, which is comparable to that of *E. gracilis* (Shibata *et al.*, 1985) and rat liver (Kobayashi *et al.*, 1981). The K_m for fumarate was 0.125 mM, a K_m comparable to that of the *E. coli* fumarase encoded by the gene *fumC* (Woods *et al.*, 1988) but higher than that for the mammalian enzymes. It is apparent that the *S. solfataricus* fumarase has a 2.5-fold greater affinity for fumarate than for malate, which is consistent with the aerobic function of this enzyme in the TCA cycle.

Since it was reported that the kinetic constants of rat liver fumarase were affected by phosphate (Kobayashi *et al.*, 1981), the effect of potassium phosphate on kinetic properties of the *S. solfataricus* enzyme was tested. Addition of 10 mM- K_3PO_4 to the test mixture resulted in an increase of K_m for malate and fumarate to 0.45 mM and 0.27 mM respectively. The inhibition of *S. solfataricus* fumarase by phosphate was less than that for rat liver fumarase, for which the addition of 10 mM- K_3PO_4 resulted in an increase of K_m from 0.013 mM to 0.33 mM for fumarate and 0.14 mM to 0.60 mM for malate (Kobayashi *et al.*, 1981).

Factors affecting enzyme activity and stability

The enzyme activity versus pH was determined over the range pH 5.0–9.0. In the range pH 6.5–8.5, fumarase exhibited 90–100% activity, with a maximum at pH 8.0. At pH 5.5 the enzyme exhibited 43%, and at pH 9.0, 70% of its maximum activity.

When enzyme activity was assayed at temperatures ranging from 40 °C to 95 °C, maximum yield of fumarate production was recorded at 85 °C, using the 10 min assay. This value for maximum catalytic activity correlates with the optimal growth temperature of *S. solfataricus*. At 95 °C the enzyme activity was still 66% of maximum, whereas at 40 °C the enzyme was almost inactive. In this respect the fumarase closely resembles other enzymes from extremely thermophilic archaeobacteria, since a number of these enzymes are inactive at temperatures below 40 °C. The effects of temperature on

Fig. 2. Thermal inactivation of fumarase from *S. solfataricus*. 0.3 µg (0.058 U) of enzyme was preincubated for 10 min (●) or 30 min (▲) in 100 mM-potassium phosphate buffer pH 7.5 at the temperatures indicated. The remaining activity was determined using the standard assay.

Table 2. Effect of organic solvents and dissociating reagents on the activity of fumarase from *S. solfataricus*

Treatment	Remaining activity (%)	
	After 10 h at 20 °C	After 14 d at 20 °C
None	100	100
Ethanol (50%, v/v)	86	83
Methanol (50%, v/v)	102	104
2-Propanol (50%, v/v)	96	85
4 M-Urea	97	57
0.1% SDS	99	1

enzyme activity are reflected in the thermostability data presented in Fig. 2. The enzyme lost no activity when incubated at 70 °C or 85 °C for 1 h prior to assay. After preincubation at 90 °C for 30 min the enzyme activity dropped to 28%, and preincubation at 100 °C for 30 min resulted in complete loss of activity.

The effect of organic solvents and dissociating reagents on enzyme activity was studied by preincubation of the purified enzyme in these reagents for 10 h or 14 d at room temperature (Table 2). After treatment with 50% (v/v) ethanol, 50% (v/v) methanol, 50% (v/v) 2-propanol, 4 M-urea or 0.1% SDS for 10 h at room temperature, no significant loss of enzyme activity was detected. Even preincubation for up to 14 d at room temperature in organic solvents resulted in only a slight loss of activity.

The relative activity of the enzyme was also determined in the organic solvents. The fumarase retained 94% of maximum activity in 40% (v/v) methanol, but only 15–20% activity in 40% (v/v) 2-propanol and 40% (v/v) ethanol when the activity was assayed in these solvents.

As expected, the fumarase of *S. solfataricus* appeared to be more thermostable and more chemostable than the fumarases of other organisms studied to date. These properties could make this enzyme attractive for use in biotechnological processes, since fumarase activity is used industrially for continuous production of L-malate (Chibata *et al.*, 1987; Yamamoto *et al.*, 1977). The usefulness of a continuous reaction process using immobilized enzyme, as used for L-malate production, is largely determined by the enzyme's stability. The enhanced chemostability of *S. solfataricus* fumarase may therefore be of relevance to this process.

Enzymic conversion of fumarate to L-malate was shown to be increased when using magnesium or calcium fumarate (Ado *et al.*, 1982); but these substrates cannot be used in an industrial process because their solubilities are very low at room temperature. The high optimum temperature and high thermostability of the *S. solfataricus* enzyme could help to solve these problems.

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