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Isolation of a thermostable enzyme catalyzing disulfide bond formation from the archaebacterium *Sulfolobus solfataricus*

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A disulfide bond-forming enzyme was purified from the cytosol of the archaebacterium *Sulfolobus solfataricus*, strain MT-4. The enzyme, assayed by its ability to oxidize and reactivate reductively denatured ribonuclease A, had a small molecular size and displayed a high thermostability. The N-terminal amino acid sequence is reported.

Archaebacteria; Disulfide bond formation; Thermostable enzyme

1. INTRODUCTION

In vivo disulfide bond formation occurs during or soon after translation. This process is significantly faster than in vitro oxidative folding systems, so the need for in vivo biocatalysts has been recognized for a long time. The best characterized system is the formation of native disulfide bonds catalyzed by protein disulfide isomerase (PDI) (reviewed in [1]). PDI, as detected in most vertebrate tissues, is a homodimer (2 × 57,000 Da) with a highly acidic pI. The formation, reduction and isomerization of disulfide bonds can be catalyzed by PDI, the product of its action depending on the initial substrates and imposed redox potential. The enzyme is usually assayed by observing the rearrangement of scrambled ribonuclease A (srRNAse, the form containing incorrectly paired disulfide bonds) to the native enzyme in the presence of a low thiol concentration.

The primary structure of PDI shows two homologous regions closely related to the thioredoxin active site, that is a redox dithiol/disulfide center formed by vicinal cysteine residues. Thioredoxin (TH) is a small (≈12,000 Da) acidic protein found in prokaryotes, yeast, plants and mammalian cells (reviewed in [2]). It is a multifunctional protein which catalyzes disulfide reduction and participates in other redox processes. Pigiet and Schuster [3] demonstrated that TH can reactivate both srRNAse and reductively denatured ribonuclease (rdRNAse), raising the possibility that TH can have an additional in vivo role in protein folding.

A metalloglycoprotein, named sulfhydryl oxidase, from bovine milk (M_r 300,000) is known to catalyze the de novo synthesis of disulfide bonds in cysteine-containing peptides and proteins [4]. A soluble sulfhydryl oxidase (M_r 66,000) which also catalyzes disulfide bond formation in proteins has been isolated from the mammalian male reproductive tract [5]. Unlike PDI, sulfhydryl oxidase does not catalyze disulfide interchange. To date, no PDI or sulfhydryl oxidase from bacterial sources have been reported.

Because disulfide bond formation is a general process for newly synthesized polypeptide chains, we searched for a protein catalyzing this event at high temperature in extracts of the extreme thermophilic archaebacterium *Sulfolobus solfataricus*, strain MT-4, which grows at 87°C. The purification of a highly thermostable enzyme which oxidizes and reactivates rdRNAse is reported here. The molecular weight and N-terminal amino acid sequence of this disulfide bond-forming (DBF) enzyme have been determined.

2. EXPERIMENTAL

2.1. *Materials*

Ribonuclease A (100 Kunitz U/mg), bovine insulin and Type III yeast RNA were obtained fro n Sigma; the protein assay reagent and materials used for SDS-PAGE were obtained from Bio-Rad. All other chemicals were highly pure.

2.2. *Protein assays*

Protein concentration was determined by the dye-binding assay of Bradford [6], using bovine serum albumin as a standard. The concentration of native RNAse was determined at 278 nm using a molar extinction coefficient of 9,700 $M^{-1} \cdot cm^{-1}$ [7]. The concentration of

Abbreviations: EDTA, ethylene diamine tetraacetic acid; DTNB, 5,5'-dithio-bis-nitrobenzoic acid; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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rdRNAse was determined at 275 nm using a molar extinction coefficient of 9,200 $M^{-1} \cdot cm^{-1}$ [8].

2.3. *Sulfhydryl concentration*

Sulfhydryl concentration was determined by reaction in 0.1 mM DTNB, 0.1 M sodium phosphate buffer, pH 7.0, and 10 mM EDTA. After a 2-min reaction, the absorbance was read at 412 nm and the concentration was calculated using a molar extinction coefficient of 13,600 $M^{-1} \cdot cm^{-1}$ [9].

2.4. *Preparation of rdRNAse substrate*

20 mg of native RNAse was dissolved in 2 ml of reducing buffer consisting of 6 M guanidine-HCl, 0.28 M 2-mercaptoethanol in 0.2 M Tris-HCl buffer, pH 9.0. The flask was flushed with N_2, sealed and left in the dark for one night at 37°C. The reduced protein was separated from the reagents by chromatography on Sephadex G-25 equilibrated with 0.01 M HCl, and stored, aliquoted at −20°C. Reaction with DTNB provided a value of 7.6 mol –SH/mol RNAse.

2.5. *Enzyme assays*

2.5.1. Ribonuclease activity.

RNAse activity was determined according to Kunitz [10], by following the decrease at 300 nm of a solution of 0.5 mg/ml yeast RNA in 50 mM sodium acetate buffer, pH 5.2, at 30°C.

2.5.2. Reoxidation and reactivation of rdRNAse.

A solution of 50 mM sodium phosphate buffer, pH 8.0, containing 15 μM rdRNAse and DBF (approx. 50 μg) was incubated at 30°C in a vial exposed to air. An identical mixture containing buffer in the place of the enzyme represented the control (air oxidation rate). At the desired times, duplicate samples were removed to measure the sulfhydryl concentration and the RNAse activity. The percent of activity regained was calculated relative to the rate obtained with an equivalent amount of native RNAse (100% activity).

2.5.3. Disulfide reduction of insulin (thioredoxin-like activity).

The catalytic reduction of insulin by DTT was measured at 30°C by the method of Holmgren [11]. DBF (approx. 100 μg) was added in 1 ml of 100 mM sodium phosphate buffer, pH 7.0, containing 2 mM EDTA and 1 mg of bovine insulin. A control cuvette contained only buffer and insulin. The reaction was started by the addition of 1 mM DTT to both cuvettes. Increasing turbidity from precipitation of insulin B chain was recorded at 650 nm.

2.6. *Purification of DBF enzymatic activity*

The extreme thermoacidophilic archaebacterium, *Sulfolobus solfataricus*, strain MT-4, was grown aerobically at 87°C and pH 3.0 [12]. 50 g of bacteria underwent freeze–thawing twice, were added to 50 g sand and 10 ml 50 mM Tris-HCl buffer, pH 8.0, containing 0.2 M NaCl, 5% glycerol, and homogenized in a Homni Mixer. The homogenate was centrifuged at 4,000 × *g* for 20 min to remove the sand: the supernatant solution was centrifuged at 160,000 × *g* for 90 min at 4°C and the residue was discarded. From a typical preparation, about 2.3 g of protein were obtained starting from 50 g of bacteria. The crude extract was stored at −20°C in aliquots of 200 mg.

2.6.1. Step 1: DEAE–Sepharose Fast Flow chromatography.

The crude extract (200 mg) was extensively dialyzed (Spectra/Por membrane tubing cut-off 3,500) against 100 vols. of 10 mM Tris-HCl buffer, pH 8.4 (Buffer A). The dialyzed solution was applied to a DEAE-Sepharose Fast Flow column (Pharmacia, 1.5×15 cm) previously equilibrated with Buffer A at 4°C. Flow rate was 30 ml/h. The flow-through, containing all DBF activity, was concentrated by a vacuum centrifuge.

2.6.2. Step 2: Mono Q FPLC chromatography

The enzyme solution (25 mg) was dialyzed overnight against Buffer A and applied in 2 separate runs to a Mono Q column (Pharmacia, 0.5×5 cm) equilibrated with the same buffer at a flow rate of 1 ml/min. All the enzymatic activity was again eluted with the equilibrating buffer. The active sample was concentrated as described above.

2.6.3. Step 3: Superdex 75 FPLC chromatography.

The concentrated sample (7 mg) was applied to a Superdex 75 High Load column (Pharmacia, 2.6×60 cm) and eluted with Buffer A supplemented with 0.2 M NaCl at a flow rate of 2 ml/min. The peak containing DBF activity (0.3 mg) was concentrated as described above.

2.7. *SDS-polyacrylamide gel electrophoresis*

SDS-PAGE [13] was performed to estimate the purity of the enzyme and to calculate its molecular weight. Samples were incubated at 100°C for 15 min in 2% SDS, 5% 2-mercaptoethanol, 5% glycerol; the run was performed using a 5% stacking gel and a 15% separating gel. The calibration kit in the range 43–2.3 kDa was supplied by Gibco BRL. Proteins were revealed by the Sigma silver staining kit.

2.8. *N-Terminal amino acid sequence*

The sample from exclusion chromatography was dialyzed against water and loaded on a C_{18} reverse-phase HPLC column (Varian, 4×150 mm) developed with a 0–80% acetonitrile gradient in 0.1% trifluoroacetic acid. The sequence analysis was performed by Edman degradation on an Applied Biosystems Mod. 477A Sequencer. The method used was that supplied by the manufacturer.

3. RESULTS

3.1. *Enzyme purification*

The enzyme (designated DBF) has been purified by a procedure which exploits its charge and molecular size. The lack of time-consuming steps allows one to obtain pure enzyme in a few days. Fig. 1 shows the SDS-PAGE analysis of the active sample after each purification step. DBF did not bind to strong anionic exchanger Mono Q at pH 8.4, thus revealing its extremely basic nature. The protein peaks eluted from DEAE and Mono Q columns by salt gradients behaved as a control when assayed for rdRNAse oxidation activity. The active sample after Mono Q displayed a marked increase of activity with respect to the preceding step. The gel filtration chromatography gave rise to almost pure DBF, as judged from SDS-PAGE. Cationic chromatography (strong or weak exchanger) performed at pH 6.0 (phosphate buffer), 7.0 or 8.0 (phosphate and Tris buffers) markedly inactivated DBF.

The elution volume of DBF on Superdex 75 column corresponded to a molecular weight below cytochrome *c*. The enzyme ran as a band of M_r 6,200 on reducing SDS-PAGE (Fig. 1).

3.2. *Kinetic studies*

The active sample was recognized throughout the purification steps by its ability to reoxidize and reactivate rdRNAse. The enzyme obtained from gel filtration chromatography was utilized for all kinetic studies. No ribonuclease activity was measured when the rdRNAse substrate was omitted from the incubation mixture. Fig. 2 shows typical data for DBF-catalyzed reoxidation and reactivation of rdRNAse at 30°C; DBF greatly en-

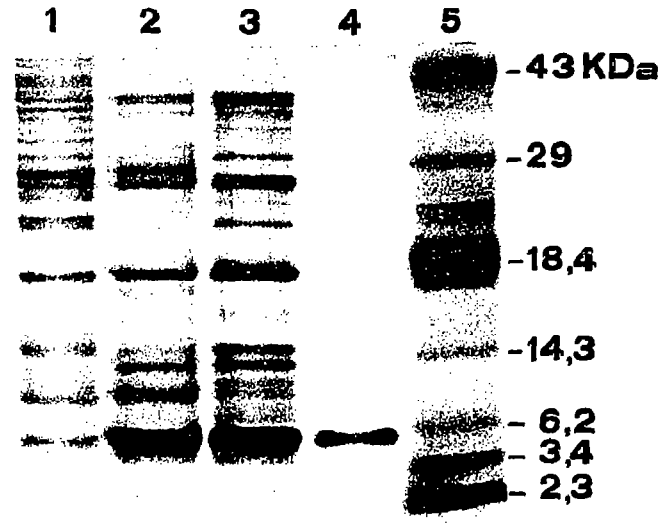

Fig. 1. SDS-PAGE analysis of various steps in the purification of DBF. Conditions were described under Experimental. The samples (2 μg/lane) were: crude extract (lane 1); DEAE-Sepharose pool (lane 2); Mono Q pool (lane 3); Superdex 75 pool (lane 4); molecular weight markers (lane 5).

hanced thiol oxidation and RNAse activity regain compared to a control. The absence of a linar relationship between the oxidation of -SH groups and RNAse activity regain is well known for the rdRNAse renaturation process. Even at room temperature DBF catalyzed a significantly faster reaction with respect to the spontaneous event (not shown). DBF was completely ineffective in catalyzing the disulfide reduction of insulin (thioredoxin-like activity).

DBF was highly thermostable: heating to 90°C for 2 h did not cause loss of activity. DBF is a real thermophilic biocatalyst in that the reaction rate increased with increasing temperature: after 30 min incubation, residual thiol groups of rdRNAse were 2 and 1.3 at 45 and 55°C, respectively, vs. 4.2 detected at 30°C (see Fig. 2).

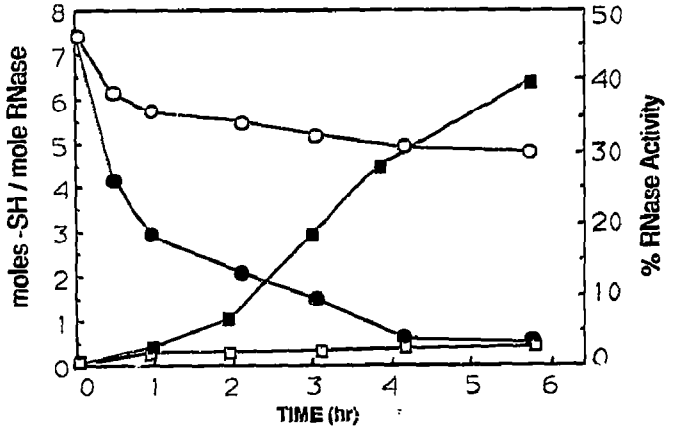

Fig. 2. Reoxidation and reactivation of rdRNAse. Conditions were described under section 2.5. Sulfhydryl groups in the absence (○) and presence (●) of DBF; RNAse activity in the absence (□) and presence (■) of DBF.

3.3. *Sequence data*

The first 12 amino acid residues of DBF were Ala-Thr-Val-Lys-Phe-Lys-Tyr-Lys-Gly-Glu-Glu-Lys. This sequence did not indicate any homology to those PDIs whose sequences have been determined. DBF shared the first 4 residues with thioredoxin isolated from *Corynebacterium nephridii* [14].

4. DISCUSSION

This communication describes the purification from the cytosol of the archaebacterium *Sulfolobus solfataricus*, strain MT-4, of a low molecular weight enzyme (designed DBF) which catalyzes the disulfide bond formation in reductively denatured RNAse. To our knowledge, this is the first report on the isolation of a disulfide bond-forming enzyme from an extreme thermophile.

DBF differed from classical PDIs in molecular size and charge. On the other hand, DBF failed to catalyze the specific thioredoxin-dependent reduction of disulfide bonds in bovine insulin. Work is under way to understand whether DBF can catalyze re-folding from randomly oxidized scrambled RNAse.

Determination of the complete primary structure of DBF is at present being carried out in order to provide further information.

DBF showed a high thermostability (complete inactivation occurred after 7 h at 90°C) and it was active even at moderate temperatures. Enzymes isolated from thermophilic microorganisms are resistant to denaturing agents [15]; in this respect, DBF displayed a good stability in the presence of urea, methanol or SDS (unpublished results).

Given its low molecular size, DBF could be a suitable tool to investigate the protein-assisted folding of thermostable proteins and, eventually, find practical application. In fact, recombinant proteins expressed in foreign hosts often accumulate in the cell in an inactive form (inclusion bodies) and enzymes like DBF can be very useful in assisting misfolded proteins to gain their active form.

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