Purification of an Anaerobic Oxidoreductase from the Hyperthermophilic Archaeon by Affinity Chromatography

M. A. SIDDIQUI* AND T. IMAI

*Department of Chemistry, University of Balochistan, Quetta, Pakistan.

[1]Department of life Science, School of life Science, Saint Paul's (Rikkyo University) Ikebukuro, Toshima-ku, Tokyo, Japan.

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Summary: *Thermococcus profundus* is a strictly anaerobic sulfur dependent archaeon that grows optimally at 80 °C by a fermentative type metabolism. During long complicated purification process, ferredoxin linked archael oxidoreductases loose much activity. Herein we report for the purification of a thermostable enzyme indolepyruvate ferredoxin oxidoreductase (IOR) from *T. profundus* by developing an effective technique of affinity chromatography. The native enzyme was purified 74-fold with a specific activity of 87 U/ mg.

Introduction

In the last two decades of 20th century various microorganism have been isolated from shallow and deep sea volcanic environments that have the remarkable property of growing at temperature near and even above 100 °C [1-6]. These hyperthermophilic organisms are classified as third domain Archaea, beside Bacteria and Eukarya [7]. Few hyperthermophilic archaeon species are methanogenic and sulfate-reducing, but the majority are sulfur dependant organisms that reduce elemental sulfur (S^0) to H_2S. Most of the S^0-dependent hyperthermophiles are strictly anaerobic sulfur reducer which grow at and above 90 °C and are heterotrophs that are obligatory dependent on elemental sulfur for growth, which they reduce by a respiratory mechanism. Only a few species of *Pyrococcus*, *Thermococcus* and *Hyperthermus* can grow well without sulfur, and they do so by fermentative type metabolism.

Like other unusual pathways [8] some unusual metabolic pathways used by hyperthermophiles for oxidation of 2-keto acids have been proposed [9-11]. Four types of extremely anaerobic fermentative oxidoreductase-type of enzymes involved in oxidation of 2-keto acids are discovered in various species of archaea [12-18]. One oxidoreductase pyruvate ferredoxin oxidoreductase (POR) [12-13] is involved in sugar metabolism, while other three indolepyruvate ferredoxin oxidoreductase (IOR) [14-18], 2-ketoisovalarate ferredoxin oxidoreductase (VOR) [15-16] and 2-ketoglutarate ferredoxin oxidoreductase (KGOR) [17] are involved in amino acids metabolism. The POR and KGOR have representative in mesophilic organisms, but IOR and VOR are unique neither found in bacteria or Eukarya.

IOR catalyzes the oxidative decarboxylation of arylpyruvates indolepyruvate, phenylpyruvate and *p*-hydroxy phenylpyruvate which originate from the aromatic amino acids tryptophan, phenylalanine, and tyrosine, respectively through the reaction of aromatic aminotransferases. The native IOR was first purified and characterized from *Pyrococcus furiosus* by Blamey et al. [13] and was first cloned and sequenced from *Thermococcus kodakaraensis* by Siddiqui et al. [18]. It has been shown that under extremely anaerobic condition by heat treatment, the separately cloned inactive α and β subunits could be partially converted to an active high molecular weight complex (195 kDa) which corresponds to the $\alpha_2\beta_2$ structure of IOR [19] and the ferredoxin is electron acceptor for IOR [13-20].

IOR was reported as highly oxygen sensitive among the ferredoxin linked oxidoreductases, and looses its activity during complicated purification process [13-18], therefore improved methods for purification of oxygen sensitive enzymes are needed. Herein a method is reported for the purification of Indolepyruvate

*To whom all correspondence should be addressed.

ferredoxin oxidoreductase (IOR) from the hyperthermophilic archaeon *Thermococcus profundus* by developing an effective technique of affinity chromatography.

Results and Discussion

Cell extracts of *T. profundus* IOR from four different batches of cells catalyzed the coenzyme A-dependent oxidation of phenylpyruvate with methyl viologen at specific rate of 1.5 to 2.0 Units/ mg. IOR activity was rapidly lost under aerobic conditions; therefore, the complete purification procedure was performed under strictly anaerobic conditions. Thus, the purification procedure was carried out under argon and all buffers contained sodium dithionite (2 mM) and DTT (2 mM) to protect against trace O_2 contamination. All buffers also contained $MgSO_4$ (1 mM) and TPP (1 mM) that seemed to stabilize IOR activity during purification. The enzyme was sensitive to inactivation by O_2, losing approximately half of its activity after exposure to air for 40 minutes at room temperature. Upon ultracentrifugation, no activity was detected in the particulate fraction, indicating that IOR is a cytoplasmic enzyme. First step for purification was performed by Q Sepharose and in second step Hydroxylapatite column was used. Hydroxylapatite column was comparatively much effective for IOR purification, but still many impurities remained. After using hydroxylapatite column many other columns were used but found not so much effective and noticed in loss of IOR activity.

Finally we designed an affinity column. α-amino toyopearl was used to bind amino group of toyopearl with COOH group of ferredoxin (purified from the same organism). Ferredoxin was used for accepting electron catalyzed by IOR during reaction. α-amino toyopearl ferredoxin complex was obtained and was used for affinity chromatography. This procedure was found very effective for affinity binding of IOR with ferredoxin and as a result 74-fold purification of IOR to apparent homogeneity with a yield of 16 % was achieved (Table- 1). About 15.5 mg of purified IOR was obtained from 120 g (wet weight) of cells with a specific activity of 87 U/ mg.

The purified enzyme gave rise to a single protein band on native gel (7 %) by both the activity staining as well as staining by coomassie brilliant blue (data not shown). Two protein bands were observed after SDS gel electrophoresis (12.5 % acrylamide) and these correspond to M_r Values of 67,000 ± 3,000 and 21,000 ± 2,000 (Fig. 1). The purified enzyme was eluted from a gel filtration column (using HPLC, Jasco) as a single protein peak with an apparent M_r of 170,000 ± 25,000.

Experimental

Materials

Bactotryptone and yeast extract were obtained from Difco Laboratories. Inorganic powder was purchased from Wako. Q Sepharose FF was obtained from Pharmacia Biotech Inc. amino TOYOPEARL was obtained from TOSO. Other chemicals were of analytical grade.

Growth of Organism

Thermococcus profundus DSM DT5432 was grown on tryptone and yeast extract with inorganic sulfur and sodium sulfide for ~10 hours at 80 °C in a medium as described previously [6].

Purification of IOR

For the purification of IOR all procedures were carried out at 25 °C under strictly anaerobic condition and in anaerobic chamber (Coy Laboratory). All buffers were made anaerobic by degassing under reduced pressure followed by concomitant bubbling of purified argon gas. The degassing followed by bubbling was repeated three times and then dithiotreitol (DTT) and sodium dithionite were added to the degassed buffers at the final concentration of 2 mM each to protect against trace O_2 contamination. The buffer A, used through

Table- 1: Purification of *T. profundus* Indolepyruvate ferredoxin oxidoreductase

Step	Activity Units	Protein mg	Specific Activity Units/mg	Recovery %	Purification Fold
Cell extract	8492	7200	1.18	100	1
Q-Sepharose	5385	1920	2.80	63.4	2.4
Hydroxylapatite	4923	660	7.6	57.9	6.4
Affinity Column	1355	15.5	87.4	16	74

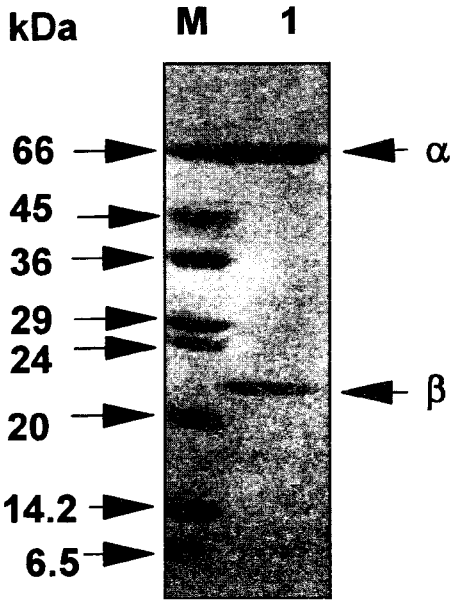

Fig. 1: The 0.1 % SDS-12.5 polyacrylamide gel electrophoresis. Samples were prepared by boiling for 3 min in a sample buffer (50 mM Tris-HCl pH 6.8, 2 % SDS, 0.1 % bromophenol blue, 10 % glycerol. M: molecular weight markers (Albumin bovine serum, 66,000; ovalbumin, 45,000; glyceraldehydes-3-phosphate dehydrogenase, 36,000; carbonic anhydrase 29,000; trypsinogen 24,000; trypsin inhibitor, 20,000; α-lactalbumin, 14,200 and Aprotenin, 6,500. Lane 1 arrows indicate α and β subunits of IOR.

out the purification was 50 mM Tris-HCl pH 8.0 containing 1 mM $MgSO_4$ and 1 mM TPP (stabilizing agent) and 0.2 M NaCl.

The frozen *T. profundus* cells were thawed with 10 mM sodium dithionite and 50 mM Tris-base. The suspension was stirred overnight with pancreatic DNase (1 mg), RNase A (1 mg) and $MgSO_4$. The homogenate was centrifuged at 80,000 x g for 40 minutes and the cell free extract 480 ml was obtained and applied to a column (5 x 50 cm) of Q Sepharose equilibrated with buffer. Column was washed with (1 liter) buffer A. IOR activity was eluted at 215-290 mM NaCl using a gradient (4 liters) from 200-600 mM NaCl in buffer A. Active fractions (400 ml) from this column were combined and directly applied onto a column (5 x 30 cm) of hydroxylapatite equilibrated with buffer A. Column was washed with (0.5 liter) buffer and than with 0.1 M potassium phosphate in the same buffer (0.5 liters). The adsorbed protein activity was eluted at 300-390 mM potassium phosphate using a gradient (2 liters) from 100-500 mM potassium phosphate in the same buffer.

Linkage of Ferredoxin with α-amino Toyopearl for Affinity Chromatography:

0.5 g α-amino toyopearl was placed in a beaker then 5 ml 0.5 M NaCl pH 5.2 was added together with 40 mg ferredoxin. 60 mg EDC (N-ethyl-N-(3-dimethylaminopropyl) carbodiamide hydrochloride) was added for condensation of amino group of amino toyopearl with carboxyl group of ferredoxin. The sample was placed at room temperature for ~40 h with shaking. Thus ferredoxin-α-amino toyopearl was obtained and filled in a column (2.5 X 10 cm). The column was washed with water then 1 M NaCl, again with water and finally with buffer A.

Active fractions of IOR (300 ml) from the previous step (hydroxylapatite) were concentrated by Amicon YM30 (for desalting and to remove low molecular weight proteins up to 30 kDa) and applied to an affinity column. Column was washed with (100 ml) buffer A. IOR exhibited activity between the ranges of 300-500 mM of NaCl by applying a gradient 1 M NaCl in the same buffer A. Active fractions with brownish colour were analyzed by native and SDS-gel electrophoresis. The purified samples were combined and concentrated by Amicon YM30 membrane and stored under anaerobic condition at -30 °C until use.

Enzyme and Protein Assay

IOR activity was determined by the phenylpyruvate dependent reduction of methyl viologen at 80°C in serum-stopped cuvetts with a rubber stopper (Aldrich, Suba seal rubber septa) under argon atmosphere. The standard assay mixture (1 ml) contained phenylpyruvate (5 mM), $MgSO_4$ (1 mM), coenzyme A (0.1 mM), thiamine pyrophosphate TPP (1 mM) and methylviologen (1.0 mM) in 50 mM TAPS buffer pH 9.0. The absorbance change at 600 nm was measured using Jasco Nihonbunko model V-530 UV/ Visible

spectrophotometer. One unit of IOR activity was defined as the oxidation of 1 µmol of phenylpyruvate per minute. The protein concentration was determined by the literature method [21] with bovine serum albumin as the standard.

Conclusions

This method is much effective for the purification of IOR. In previous report less amount of IOR was obtained with specific activity of 38 U/ mg (Mai and Adams, 1994), but with this method 74-fold purification was achieved with a specific activity of 87 U/ mg.

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