Cloning, sequence, and properties of the soluble pyridine nucleotide transhydrogenase of Pseudomonas fluorescens

Citation for published version:

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published in:
Journal of Bacteriology

Publisher Rights Statement:
Free in PMC.

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
Cloning, Sequence, and Properties of the Soluble Pyridine Nucleotide Transhydrogenase of *Pseudomonas fluorescens*

Christopher E. French, Birgitte Boonstra, Karen A. J. Bufton, and Neil C. Bruce*

Institute of Biotechnology, University of Cambridge, Cambridge CB2 1QT, United Kingdom

Received 27 November 1996/Accepted 3 February 1997

The gene encoding the soluble pyridine nucleotide transhydrogenase (STH) of *Pseudomonas fluorescens* was cloned and expressed in *Escherichia coli*. STH is related to the flavoprotein disulfide oxidoreductases but lacks one of the conserved redox-active cysteine residues. The gene is highly similar to an *E. coli* gene of unknown function.

Pyridine nucleotide transhydrogenases catalyze the transfer of reducing equivalents between NAD and NADP pools. A membrane-bound, proton-pumping transhydrogenase, specific for the 4A proton of NADH and the 4B proton of NADPH, occurs in mitochondria and in some bacteria, such as *Escherichia coli*, and has been studied in some detail (3, 8). This enzyme couples proton import with oxidation of NADH and reduction of NADP⁺, and its physiological role is believed to be production of NADPH for reductive biosyntheses. Less well-known is a soluble transhydrogenase (STH) reported to occur in *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, and *Azotobacter vinelandii* (13). This enzyme is a flavoprotein specific for the 4B protons of both NADH and NADPH. STH is not energy dependent but is strongly inhibited by NADP⁺, suggesting that its physiological role is the conversion of NADPH generated by peripheral catabolic pathways in these bacteria to NADH, which can be oxidized for energy generation (16). STH is remarkable for its formation of large polymers. The subunit *M*_s is approximately 54,000, whereas the minimal active form of the *P. aeruginosa* enzyme has an *M*_s of approximately 1.6 million (18). This minimal form further aggregates on isolation to form filaments of lengths exceeding 500 nm (9). The enzyme from *A. vinelandii* displays similar behavior, although the structure of the filaments appears to be different (15). STH also shows interesting kinetic behavior, with activity strongly activated by NADPH and 2'-AMP and inhibited by NADP⁺ (19). The presence of Ca²⁺ favors activation and reduces inhibition.

To gain some insight into the structural basis for the aggregation and regulation of this unusual enzyme, we sought to clone the gene encoding STH from *P. fluorescens* NCIMB9815, a close relative of *P. aeruginosa*.

**Purification of STH from *P. fluorescens***. STH activity was assayed by following the reduction of thionicotinamide adenine dinucleotide (tNAD⁺) at 400 nm in a reaction mixture consisting of 0.1 mM NADPH and 0.1 mM tNAD⁺ (Sigma Chemical Co.) in 50 mM Tris-HCl buffer (pH 7.0). The molar extinction coefficient of tNADH at 400 nm was taken as 11,300 liters mol⁻¹ cm⁻¹ (2). One unit of enzyme activity was defined as that amount of activity reducing 1 μmol of tNAD⁺ per min under these conditions.

STH was purified from cells of *P. fluorescens* NCIMB9815 according to a modification of the method of Höjeberg et al. (7). Cells were grown to stationary phase in 1 liter of SOB medium (14). The cells were harvested by centrifugation (5,000 × g for 15 min) and resuspended in 20 ml of buffer A (50 mM Tris-HCl [pH 7.0] with 2 mM dithiothreitol). The cells were then disrupted by sonication (25 bursts of 5 s at 12 μm separated by 30-s pauses for cooling in an ice-water bath) with an MSE Soniprep 150. Cell debris was removed by centrifugation (25,000 × g for 10 min). The extract contained 93 U of STH activity at a specific activity of 0.19 U/mg.

STH was purified by using a column with an inner diameter of 1 cm, packed with 6 ml of adenosine-2',5'-diphosphate (Sigma) (Sigma Chemical Co.). The column was operated at 12 ml/h during loading and at 24 ml/h during washing. All procedures were performed at 4°C, and all buffers contained 2 mM dithiothreitol. After equilibration of the column with 5 mM CaCl₂ in buffer A, crude extract (20 ml), to which CaCl₂ had been added to a final concentration of 5 mM, was loaded onto the column. The column was then washed with 90 ml of 0.4 M NaCl–5 mM CaCl₂ in buffer A, followed by 24 ml of 0.7 M NaCl–5 mM CaCl₂ in buffer A. Bound STH was eluted with 50 mM Tris-HCl (pH 8.9) containing 0.4 M NaCl. Active fractions (15 ml) were pooled. The pooled product was concentrated by ultrafiltration with an Amicon 8050 ultrafiltration cell fitted with a membrane with a nominal *M*_s cutoff of 10,000 and was then dialyzed with buffer A to reduce the pH and salt concentration. The final volume was 1.5 ml. This material contained 62 U of STH activity at a specific activity of 140 U/mg.

The product was then applied to a gel filtration column with an inner diameter of 1.6 cm, packed with 150 ml of Sephacryl S-300 (packed height, 75 cm) (Pharmacia) equilibrated with buffer A. The column was operated at 8 ml/h. Active fractions (16 ml) were pooled and concentrated by ultrafiltration as described above to a final volume of 1 ml. The product contained 26 U of STH activity at a specific activity of 310 U/mg, an overall 1,630-fold purification.

Prior to analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the sample was further concentrated by freeze-drying and resuspension in a small volume of buffer A. The reconstituted material was not active. SDS-PAGE showed a single protein band with an apparent *M*_s of approximately 54,000, consistent with the value reported for the enzyme from *P. aeruginosa* (18).

**Cloning of the sfh gene**. The N-terminal sequence of STH was determined by automated Edman degradation and was found to be A-V-Y-N-Y-D-V-V-L-G-S-(G/V)-P-A-G-E-(G/
Based on the codon bias of *P. fluorescens* genes in these sequence databases, the following degenerate oligonucleotide was designed: AC-(C/G)AC-(C/G)AC-GTC-GTA-GTT-GTA-(C/G)AC-(G/C)GC (based on residues 1 to 9 of the N-terminal sequence).

Southern blotting and cloning procedures were performed according to standard methods (14). Southern blots of genomic DNA from *P. fluorescens* NCIMB9815 showed that this oligonucleotide bound most strongly to a 5.0-kb EcoRI fragment. This fragment was cloned in pBluescript SK

_v._ The recombinant plasmids were designated pSTH-G1 and pSTH-G2. The gene _sth_ was localized by restriction mapping of the insert followed by Southern blot analysis with the oligonucleotide probe. Sequencing indicated the presence of an open reading frame encoding a protein of the same N-terminus sequence as that determined for STH. Various subclones were prepared in pBluescript SK+ and sequenced by using vector-based primers. The sequence of _sth_ and the deduced amino acid sequence of STH are shown in Fig. 1. The gene encodes a protein of 463 residues with an _M_ of 50,876, as determined by the Genetics Computer Group (GCG) PEP-TIDESORT program (4), excluding the initiating methionine. This is slightly lower than the _M_ expected from the migration position in SDS-PAGE.

**Expression of STH in _E. coli_.** Cell extracts prepared from saturated cultures of *E. coli* JM109/pSTH-G1 or pSTH-G2 showed detectable STH activity, assayed by the reduction of NAD₆ in the presence of NADPH. A 1.5-kb SacII/XhoI fragment from pSTH-G1 was subcloned in pBluescript SK+. This plasmid was designated pSTH1. In pSTH1, _sth_ is in the correct orientation to be expressed from the lac promoter of pBluescript SK+. Cell extracts from saturated cultures of *E. coli* JM109/pSTH1 in the absence or presence of 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside) showed transhydrogenase activity of 4.1 or 22.0 U/mg, respectively. Based on the specific activity of purified STH, it was estimated that in the latter case STH formed approximately 6% of soluble cell protein, approximately 100 times the level seen in _P. fluorescens_. The recombinant STH was purified to apparent homogeneity in a single affinity chromatography step by using adenosine-2',5'-diphosphate agarose. Cell extract was prepared as described above from 1 liter of saturated culture of *E. coli* JM109/pSTH1 grown in the presence of 0.4 mM IPTG. Of the resulting 25 ml of cell extract, 5 ml, containing 2,140 U of STH, was applied to a 2 ml column of an adenosine diphosphate agarose resin and eluted with an 8-ml gradient from 50 mM Tris-HCl (pH 8.0) to 200 mM Tris-HCl (pH 8.0). The protein peak was pooled and concentrated using a Centricon 10 device. The active fraction was then applied to a HiPrep 26/60 desalting column equilibrated with 5% (v/v) glycerol in 20 mM Tris-HCl (pH 8.0). The STH activity was eluted with a single peak of 50% (v/v) glycerol in 20 mM Tris-HCl (pH 8.0) and concentrated as described above. The protein was analyzed by SDS-PAGE and Western blot analysis using anti-STH antibody. The purified STH was used in further experiments.
activity at a specific activity of 27 U/mg, was loaded onto a column packed with adenosine-2',5'-diphosphate agarose as described above. The column was washed with 35 ml of 0.7 M NaCl-5 mM CaCl₂ in buffer A. STH was then eluted with 0.4 M NaCl in 50 mM Tris-HCl (pH 8.9). The most active fractions, totalling 13 ml, were pooled, concentrated, and diafiltered as described above, except that a membrane with a nominal $M_c$ cutoff of 300,000 was used. The product contained 900 U of STH activity at a specific activity of 300 U/mg. This material appeared to be homogeneous by SDS-PAGE; the gel filtration step was therefore omitted. The purified STH was stored at −20°C in buffer A with 2 mM dithiothreitol, with no detectable loss of activity over several weeks. Storage over several months resulted in great loss of activity, which could be reversed by incubation with fresh dithiothreitol. The N-terminal sequence of the recombinant enzyme was determined and was found to be identical to that determined for the enzyme purified from P. fluorescens.

**Characterization of recombinant STH.** The properties of the recombinant STH were compared to those reported for the enzyme from P. aeruginosa. The subunit $M_c$ as determined by SDS-PAGE is consistent with that previously reported (13, 18).
To determine whether the recombinant enzyme was capable of forming large polymers, samples were adsorbed to glow discharged carbon Formvar films from a solution of 1 mg of protein/ml, negatively stained with 1% (wt/vol) uranyl acetate, and examined by transmission electron microscopy with a Philips CM100 electron microscope operated at 80 kV. Long polymers of approximately 10 nm in diameter and in excess of 500 nm in length were observed (Fig. 2). This is consistent with previous reports (9) and shows unequivocally that only one type of subunit is required for polymer formation.

**Sequence comparisons.** The deduced amino acid sequence of STH was compared to other sequences in the OWL database. It was found that STH is highly similar in sequence to the flavoprotein disulfide oxidoreductases, particularly dihydrolipoamide dehydrogenases, mercuric ion reductases, glutathione reductases, and trypanothione reductases. The most similar sequence detected was that of an uncharacterized dehydrogenase from *E. coli* (6), encoded by the *udhA* gene, which showed 60% sequence identity and 77% similarity, as determined by the GCG GAP program (4). Various dihydrolipoamide dehydrogenases showed up to 31% identity and 52% similarity. A multiple sequence alignment of several related proteins is shown in Fig. 3. It is noteworthy that STH lacks one of the conserved cysteine residues which form the redox-active disulfide bond characteristic of this family of enzymes. A less closely related enzyme, the NADH peroxidase of *Enterococcus faecalis*, has been shown to possess a unique redox center consisting of a single stabilized cysteine-sulfenic acid residue (11); however, it seems likely that no redox center other than the flavin is required in STH, which appears to operate by a simple ping-pong mechanism (19).

The recombinant STH did not show significant reduction of lipoamide (<0.2 U/mg) in an assay system consisting of 0.2 mM NADPH or NADH and 0.2 mM lipoamide in 50 mM Tris-HCl buffer (pH 7.0) at 30°C. Under these conditions, dihydrolipoamide dehydrogenase from bovine intestinal mucosa (Sigma) displayed vigorous activity (100 U/mg) with NADH but no significant activity (<0.1 U/mg) with NADPH. Lack of dihydrolipoamide dehydrogenase activity in STH is consistent with earlier reports (2).

Glutathione reductases, trypanothione reductases, and mercuric ion reductases are active as homodimers (21). Dihydrolipoamide dehydrogenases form a part of several multienzyme complexes, including pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (E3 component), branched-chain α-keto acid dehydrogenase, and the glycine cleavage system (L-chin). In these complexes they form homodimers which interact with other components. The enzymes of this family consist of three domains: an N-terminal flavin-binding domain, a central NAD(P)-binding domain, and a C-terminal dimerization domain. The N-terminal and central domains show considerable similarity and may have evolved through gene duplication (10). The relationship of STH to these enzymes casts no obvious light upon the polymerization of STH or its apparent binding of adenine nucleotides at a second, regulatory site in addition to the catalytic site (7). Conceivably, certain of the binding sites in the active 30-subunit form act as regulatory sites and functions as active sites. Such a mechanism was earlier proposed to account for incomplete reduction of flavin by reduced cofactors (2). In this regard it is interesting that negative co-

FIG. 3. Sequence alignment of STH and selected related enzymes. This alignment was generated by using the PILEUP program of the GCG package. The enzymes shown are as follows (with OWL database accession numbers in parentheses): sth_psefl, soluble transhydrogenase from *P. fluorescens* (this study); udha_ecoli, uncharacterized dehydrogenase from *E. coli* (P27306) (6); gshr_ecoli, glutathione reductase from *E. coli* (P06715) (5); dldh_psefl, dihydrolipoamide dehydrogenase from *P. fluorescens* (P14218) (1); mera_bacsr, mercuric ion reductase from *Bacillus* sp. strain RC067 (P16171) (17); nape_enfla, NADH peroxidase from *E. fucalis* (P37062) (12). Indicated in bold type are the redox-active cysteine residues and the Rossmann fold Gly-X-Gly-X-(Gly/Ala) motifs forming the flavin adenine dinucleotide and NAD(P) binding sites (10, 20).
operativity in cofactor binding has been reported for dihydro-lipoamide dehydrogenase and glutathione reductase (21). We hope to address these questions through structural studies of the recombinant enzyme. The availability of large amounts of STH will also enable us to investigate the use of this enzyme for cofactor cycling in biotransformation processes dependent on both NAD and NADP.

We gratefully acknowledge the assistance of Jeremy Skepper (Department of Anatomy, University of Cambridge) with electron microscopy and of Richard Williams (Institute of Biotechnology, University of Cambridge) with figure preparation.

REFERENCES