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Cloning, Sequence, and Properties of the Soluble Pyridine Nucleotide Transhydrogenase of *Pseudomonas fluorescens*

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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_r is approximately 54,000, whereas the minimal active form of the *P. aeruginosa* enzyme has an M_r 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 Ca^{2+} 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 agarose (packed height, 7.6 cm) (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 $CaCl_2$ in buffer A, crude extract (20 ml), to which $CaCl_2$ 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 $CaCl_2$ in buffer A, followed by 24 ml of 0.7 M NaCl–5 mM $CaCl_2$ 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_r cutoff of 10,000 and was then diafiltered 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.

This 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_r of approximately 54,000, consistent with the value reported for the enzyme from *P. aeruginosa* (18).

Cloning of the *sth* 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-V-L-G-S-(G/V)-P-A-G-E-(G/

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-208  ACGGGCGGTG AAACGTAACG AGTGCTGTGT AGTAAAGGCA AAACTGGCTA    -159

-158  CGGAACGCGA CCAAGGGTTA ATGTGCGCGG CGTTGAGCCT TATATAGACT    -109

-108  GTGCCCCGGT TCAACACACT TGAGCCAAAT TGTCCTTCAT GACCGCTGGC     -59

          SacII
 -58  CGCGGCATGA TTTAGCCAGG CGTCCAACCG TACGCCTGGC CTGTTTTGAG     -9

  -8  GAGTACGC ATG GCT GTC TAC AAC TAC GAC GTG GTG GTA CTG GGT TCC   39
            Met Ala Val Tyr Asn Tyr Asp Val Val Val Leu Gly Ser
             -1   1              5                  10

  40  GGC CCG GCT GGA GAA GGT GCG GCG ATG AAC GCC GCG AAG GCA GGG    84
      Gly Pro Ala Gly Glu Gly Ala Ala Met Asn Ala Ala Lys Als Gly
                15                  20                  25

  85  CGC AAG GTG GCG ATG GTC GAT AGC CGT CGC CAG GTC GGC GGT AAC   129
      Arg Lys Val Ala Met Val Asp Ser Arg Arg Gln Val Gly Gly Asn
                30                  35                  40

 130  TGC ACC CAC CTG GGT ACC ATC CCG TCC AAG GCA TTG CGT CAC TCC   174
      Cys Thr His Leu Gly Thr Ile Pro Ser Lys Ala Leu Arg His Ser
                45                  50                  65

 175  GTT CGC CAG ATC ATG CAG TTC AAC ACC AAC CCG ATG TTC CGG GCC   219
      Val Arg Gln Ile Met Gln Phe Asn Thr Asn Pro Met Phe Arg Ala
                60                  65                  70

 220  ATT GGC GAG CCG CGC TGG TTC TCG TTC CCG GAT GTG TTG AAA AGC   264
      Ile Gly Glu Pro Arg Trp Phe Ser Phe Pro Asp Val Leu Lys Ser
                70                  75                  80

 265  GCT GAA AAA GTC ATC TCC AAG CAA GTC GCC TCG CGT ACC GGC TAC   309
      Ala Glu Lys Val Ile Ser Lys Gln Val Ala Ser Arg Thr Gly Tyr
                85                  90                  95

 310  TAC GCC CGT AAC CGC GTC GAC CTG TTC TTC GGT ACC GGC AGC TTC   354
      Tyr Ala Arg Asn Arg Val Asp Leu Phe Phe Gly Thr Gly Ser Phe
               100                 105                 110

 355  GCC GAC GAG CAA ACC GTC GAG GTG GTC TGC GCC AAT GGC GTG GTC   399
      Ala Asp Glu Gln Thr Val Glu Val Val Cys Ala Asn Gly Val Val
               115                 120                 125

 400  GAG AAA CTG GTG GCC AAG CAC ATC ATC ATT GCC ACC GGC TCG CGC   444
      Glu Lys Leu Val Ala Lys His Ile Ile Ile Ala Thr Gly Ser Arg
               130                 135                 140

 445  CCG TAT CGC CCG GCG GAT ATC GAT TTC CAC CAC CCA CGT ATC TAC   489
      Pro Tyr Arg Pro Ala Asp Ile Asp Phe His His Pro Arg Ile Tyr
               145                 150                 155

 490  GAT AGC GAT ACC ATC CTC AGC CTG GGC CAC ACC CCA CGC AAA CTG   534
      Asp Ser Asp Thr Ile Leu Ser Leu Gly His Thr Pro Arg Lys Leu
               160                 165                 170

 535  ATC ATC TAT GGC GCC GGC GTC ATT GGC TGT GAA TAC GCC TCG ATC   579
      Ile Ile Tyr Gly Ala Gly Val Ile Gly Cys Glu Tyr Ala Ser Ile
               175                 180                 185
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FIG. 1. Sequence of *sth* and deduced amino acid sequence of STH. Both strands were sequenced over the region shown. Nucleotides are numbered, starting with 1 for the A of the initiating ATG.

V)-A-A-M-N-A-A-(R/D); parentheses indicate uncertain assignments.

Based on the codon bias of *P. fluorescens* genes in the 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 *Eco*RI fragment. This fragment was cloned in pBluescript SK+ in both orientations. 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-terminal 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_r of 50,876, as determined by the Genetics Computer Group (GCG) PEPTIDESORT program (4), excluding the initiating methionine. This is slightly lower than the M_r expected from the migration position in SDS-PAGE.

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 580  TTC AGC GGC CTG GGT GTG CTG GTC GAG CTG GTC GAC AAC CGC GAC   624
      Phe Ser Gly Leu Gly Val Leu Val Glu Leu Val Asp Asn Arg Asp
                190                 195                 200

 625  CAG TTG CTG AGC TTC CTC GAC TCG GAA ATC TCC CAG GCG TTG AGC   669
      Gln Val Leu Ser Phe Leu Asp Ser Glu Ile Ser Glu Ala Leu Ser
                205                 210                 215

 670  TAC CAC TTC AGC AAC AAC AAC ATC ACT GTG CGC CAT AAC GAA GAG   714
      Tyr His Phe Ser Asn Asn Asn Ile Thr Val Arg His Asn Glu Glu
                220                 225                 230

 715  TAC GAT CGG GTC GAA GGC CTG GAC AAC GGG GTG ATC CTG CAC CTC   759
      Tyr Asp Arg Val Glu Gly Leu Asp Asn Glu Val Ile Leu His Leu
                235                 240                 245

 760  AAG TCC GGC AAG AAG ATC AAG GCC GAC GCC TTG CTG TGG TGC AAC   804
      Lys Ser Gly Lys Lys Ile Lys Ala Asp Ala Leu Leu Trp Cys Asn
                250                 255                 260

 805  GGT CGT ACC GGC AAC ACC GAC AAG CTG GGC ATG GAA AAC ATC GGG   849
      Gly Arg Thr Gly Asn Thr Asp Lys Leu Gly Met Glu Asn Ile Gly
                265                 270                 275

 850  GTC AAG GTC AAC AGC CGT GGC CAG ATC GAG GTG GAC GAA AAC TAC   894
      Val Lys Val Asn Ser Arg Gly Gln Ile Glu Val Asp Glu Asn Tyr
                280                 285                 290

 895  CGC ACC TGT GTG ACC AAC ATC TAT GGC GCC GGT GAC GTG ATC GGC   939
      Arg Thr Cys Val Thr Asn Ile Tyr Gly Ala Gly Asp Val Ile Gly
                295                 300                 305

 940  TGG CCG AGC CTG GCC AGT GCC GCC CAT GAC CAG GGC CGT TCG GCC   984
      Trp Pro Ser Leu Ala Ser Ala Ala His Asp Gln Gly Arg Ser Ala
                310                 315                 320

 985  GCT GGC AGC ATC GTC GAC AAC GGC AGC TGG CGC TAT GTG AAC GAC  1029
      Ala Gly Ser Ile Val Asp Asn Gly Ser Trp Arg Tyr Val Asn Asp
                325                 330                 335

1030  GTA CCG ACC GGG ATC TAC ACG ATT CCG GAG ATC AGC TCG ATC GGC  1074
      Val Pro Thr Gly Ile Tyr Thr Ile Pro Glu Ile Ser Ser Ile Gly
                340                 345                 350

1075  AAG AAC GAA CAC GAA CTG ACC AAG GCC AAG GTG CCT TAC GAA GTG  1119
      Lys Asn Glu His Glu Leu Thr Lys Ala Lys Val Pro Tyr Glu Val
                355                 360                 365

1120  GGC AAG GCG TTC TTC AAG AGC ATG GCG CGT GCG CAG ATC GCC GGT  1164
      Gly Lys Ala Phe Phe Lys Ser Met Ala Arg Ala Gln Ile Ala Gly
                370                 375                 380

1165  GAG CCG CAA GGC ATG CTG AAG ATC CTG TTT CAC CGC GAG ACC CTG  1209
      Glu Pro Gln Gly Met Leu Lys Ile Leu Phe His Arg Glu Thr Leu
                385                 390                 395

1210  GAA GTC CTC GGC GTG CAT TGC TTC GGC TAC CAG GCT TCG GAG ATC  1254
      Glu Val Leu Gly Val His Cys Phe Gly Tyr Gln Ala Ser Glu Ile
                400                 405                 410

1255  GTG CAC ATC GGC CAG GCC ATC ATG AAC CAG CCG GGC GAG CAA AAT  1299
      Val His Ile Gly Gln Ala Ile Met Asn Gln Pro Gly Glu Gln Asn
                415                 420                 425

1300  ACC CTC AAG TAT TTC GTC AAC ACC ACC TTC AAC TAC CCG ACC ATG  1344
      Thr Leu Lys Tyr Phe Val Asn Thr Thr Phe Asn Tyr Pro Thr Met
                430                 435                 440

1345  GCC GAA GCC TAT CGG GTA GCG GCC TAC GAT GGC CTC AAC CGG CTT  1389
      Ala Glu Ala Tyr Arg Val Ala Ala Tyr Asp Gly Leu Asn Arg Leu
                445                 450                 455

1390  TTT TGA GCGGCTCCGG CCGGTGGCCT GAGCCGGCCG GGGAGACCGA TTTCAGTAA  1443
      Phe  *

         XhoI
1444  TTCTCGAG
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FIG. 1—*Continued.*

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 $tNAD^+$ in the presence of NADPH. A 1.5-kb *Sac*II/*Xho*I 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

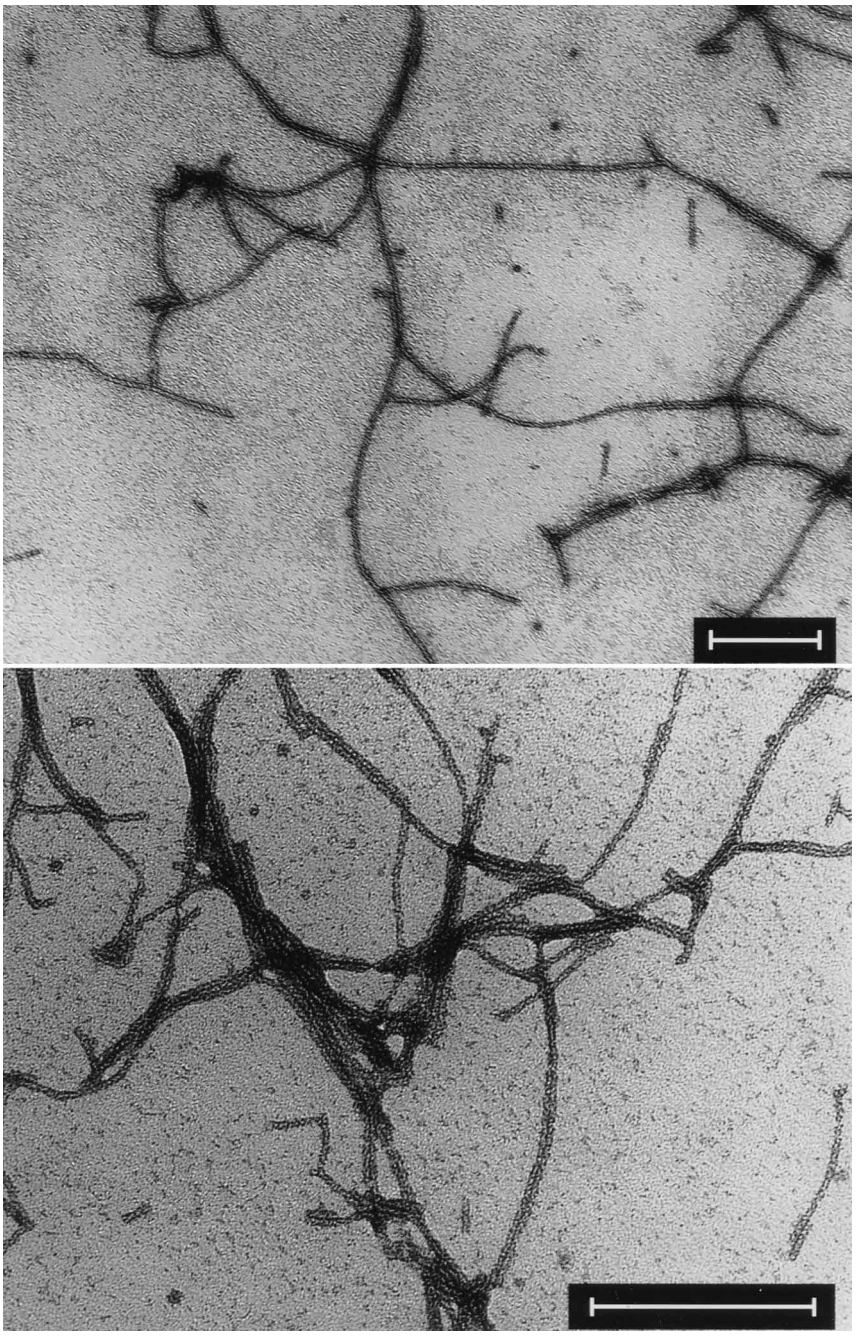

FIG. 2. Electron micrographs showing formation of filaments by purified STH. STH was adsorbed onto glow discharged carbon Formvar films from a 1.0-mg/ml solution in 50 mM Tris-HCl buffer (pH 7.0). Grids were then negatively stained with 1% (wt/vol) uranyl acetate and examined with a Philips CM100 electron microscope at 80 kV. Bars, 200 nm.

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 $CaCl_2$ 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_r 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_r as determined by SDS-PAGE is consistent with that previously reported (13, 18).

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           1                                                   50
mera_bacsr MKKYRVNVQG MTCSGCEQHV AVALENMGAK AIEVDFRRGE AVFELPDDVK

           51                                                  100
mera_bacsr VEDAKNAIAD ANYHPGEAEE FQSEQKTNLL KKYRLNVEGM TCTGCEEHIA

           101                                                 150
mera_bacsr VALENAGAKG IEVDFRRGEA LFELPYDVDI DIAKTAITDA QYQPGEAEEI

           151                                                 200
 sth_psefl ..........  .....AVYNY DVVVLGSGPA GEGAAMNAAK AGRKVAMVDS
udha_ecoli ..........  .......... ..........  .....MGLVK QGARVAVIER
dldh_psefl ..........  ......SQKF DVVVIGAGPG GVVAAIRAAQ LGLKTACIEK
mera_bacsr QVQSEKRTDV  SLNDEGNYDY DYIIIGSGGA AFSSAIEAVA LNAKVAMIE.
gshr_ecoli ..........  .....MTKHY DYIAIGGGSG GIASINRAAM YGQKCALIEA
nape_entfa ..........  .........M KVIVLGSSHG GYEAVEELLN LHPDAEI...

           201                                                 250
 sth_psefl ......RRQV GGNCTHLGTI PSKALRHSVR QIMQFNTNPM FRAI.GEPRW
udha_ecoli ......YQNV GGGCTHWGTI PSKALRHAVS RIIEFNQNPL YSDH.SRLLR
dldh_psefl YIGKEGKVAL GGTCLNVGCI PSKALLDSSY KYHEAKEAFK VHGIEAKGVT
mera_bacsr ......RGTV GGTCVNVGCV PSKTLLRAGE INHLAKNNPF V.GLHTSASN
gshr_ecoli .......KEL GGTCVNVGCV PKKVMWHAAQ IREAIHMYGP DYGFDTTINK
nape_entfa ..........  ..........  ..........  ...QWYEKGD FISFLSCGMQ

           251                                                 300
 sth_psefl FSFPDVLKSA EKV...ISKQ VASR.TGYYA RNRVDLFFGT GSFADEQTVE
udha_ecoli SSFADILNHA DNV...INQQ TRMR.QGFYE RNHCEILQGN ARFVDEHTLA
dldh_psefl IDVPAMVARK ANI...VKNL TGG.IATLFK ANGVTSFEGH GKLLANKQVE
mera_bacsr VDLAPLVKQK NDL...VTEM RNEKYVNLID DYGFELIKGE SKFVNENTVE
gshr_ecoli FNWETLIASR TAY...IDRI HTS.YENVLG KNNVDVIKGF ARFVDAKTLE
nape_entfa LYLEGKVKDV NSVRYMTGEK MESRGVNVFS NTEITAIQP. ....KEHQVT

           301                                                 350
 sth_psefl VVCANGVVEK LVA.KHIIIA TGSRPYRPAD IDFHHPRIYD SDTIL.....
udha_ecoli LDCPDGSVET LTA.EKFVIA CGSRPYHPTD VDFTHPRIYD SDSIL.....
dldh_psefl VTGLDGKTQV LEA.ENVIIA SGSRPVEIPP APLSDDIIVD STGAL.....
mera_bacsr VNGNQ..... ITA.KRFLIA TGASSTAPNI PGLDEVDYLT STSLL.....
gshr_ecoli VNG.....ET ITA.DHILIA TGGRPSHPDI PGVEYG..ID SDGFF.....
nape_entfa VKDLVSGEER VENYDKLIIS PGAVPFELDI PGKDLDNIYL MRGRQWAIKL

           351                                                 400
 sth_psefl ...SLGHTPR KLIIYGAGVI GCEYASIFSG LGVLVELVDN RDQLLS.FLD
udha_ecoli ...SMHHEPR HVLIYGAGVI GCEYASIFRG MDVKVDLINT RDRLLA.FLD
dldh_psefl ...EFQAVPK KLGVIGAGVI GLELGSVWAR LGAEVTVLEA LDKFLP.AAD
mera_bacsr ...ELKKVPN RLTVIGSGYI GMELGQLFHN LGSEVTLIQR SERLLK.EYD
gshr_ecoli ...ALPALPE RVAVVGAGYI AVELAGVING LGAKTHLFVR KHAPLR.SFD
nape_entfa KQKTVDPEVN NVVVIGSGYI GIEAAEAFAK AGKKVTVIDI LDRPLGVYLD

           401                                                 450
 sth_psefl SEISQALSYH FSNNNITVRH NEEYDRVEGL DNGVILHLK. ......SGKK
udha_ecoli QEMSDSLSYH FWNSGVVIRH NEEYEKIEGC DDGVIMHLK. ......SGKK
dldh_psefl EQIAKEALKV LTKQGLNIRL GA...RVT.A SEVKKKQVTV TFTDANGEQK
mera_bacsr PEISEAITKA LTEQGINLVT GATYERVEQD GDIKKVHVEI NGKKRIIEA.
gshr_ecoli PMISETLVEV MNAEGPQLHT NAIPKAV... ..VKNTDGSL TL.ELEDGRS
nape_entfa KEFTDVLTEE MEANNITIAT GETVERYEGD GRVQKV.... ....VTDKNA

           451                                                 500
 sth_psefl IKADALLWCN GRTGNTDKLG MENIGVKVNS RGQIEVDENY RTCVTNIYGA
udha_ecoli LKADCLLYAN GRTGNTDSLA LQNIGLETDS RGQLKVNSMY QTAQPHVYAV
dldh_psefl ETFDKLIVAV GRRPVTTDLL AADSGVTLDE RGFIYVDDHC KTSVPGVFAI
mera_bacsr ...EQLLIAT GRKPIQTSLN LHAAGVEVGS RGEIVIDDYL KTTNSRIYSA
gshr_ecoli ETVDCLIWAI GREPANDNIN LEAAGVKTNE KGYIVVDKYQ NTNIEGIYAV
nape_entfa YDADLVVVAV GVRPNTAWL. ..KGTLELHP NGLIKTDEYM RTSEPDVFAV

           501                                                 550
 sth_psefl GDVI...... ....GWPSLA SAAHDQGRSA AGSIV.DNGS WRY.VNDVPT
udha_ecoli GDVI...... ....GYPSLA SAAYDQGRIA AQALVKGEAT AHL.IEDIPT
dldh_psefl GDVV...... ....RGAMLA HKASEEGVMV A.ERIAG.HK AQMNYDLIPS
mera_bacsr GDVT...... ....PGPQFV YVAAYEGGLA ARNAIGG.LN QKVNLEVVPG
gshr_ecoli GDNT...... ....GAVELT PVAVAAGRRL SERLFNNKPD EHLDYSNIPT
nape_entfa GDATLIKYNP ADTEVNIALA TNARKQGRFA VKN.LEEPVK PFPGVQGSSG

           551                                                 600
 sth_psefl GIYTIPEISS IGKNEHELTK AKVPYEV..G KAFFKSMARA QIAGEPQGML
udha_ecoli GIYTIPEISS VGKTEQQLTA MKVPYEV..G RAQFKHLARA QIVGMNVGTL
dldh_psefl VIYTHPEIAW VGKTEQTLKA E..GVEVNVG TFPFAASGRA MAANDTTGLV
mera_bacsr VTFTSPSIAT VGLTEQQAKE K..GYEVKTS VLPLDAVPRA LVNRETTGVF
gshr_ecoli VVFSHPPIGT VGLTEPQARE QYGDDQVKVY KSSFTAMYTA VTTHRQPCRM
nape_entfa LAVFDYKFAS TGINE..VMA QKLGKETKAV TV.VEDYLMD FNPDKQKAWF

           601                                                 650
 sth_psefl KILFHRETLE VLGVHCFGYQ ASEIVHIGQA IMNQPGEQNT LKYFVNTTFN
udha_ecoli KILFHRETKE ILGIHCFGER AAEIIHIGQA IMEQKGGGNT IEYFVNTTFN
dldh_psefl KVIADAKTDR VLGVHVIGPS AAELVQQGAI GME...FGTS AEDLGMMVFS
mera_bacsr KLVADAKTLK VLGAHVVAEN AGDVIYAATL AVK...FGLT VGDLRETMAP
gshr_ecoli KLVCVGSEEK IVGIHGIGFG MDEMLQGFAV ALK...MGAT KKDFDNTVAI
nape_entfa KLVYDPETTQ ILGAQLMS.. KADLTANINA ISLAIQAKMT IEDLAYADFF

           651                              682
 sth_psefl YPTMAEA... .YRVAAYDGL NRLF...... ..
udha_ecoli YPTMAEA... .YRVAALNGL NRLF...... ..
dldh_psefl HPTLSEA... .LHEAALAVN GHAIHIANRK KR
mera_bacsr YLTMAEG... .LKLAVLTFD KDVSKLSCCA G.
gshr_ecoli HPTAAEE... .FVTMR.... .......... ..
nape_entfa FQPAFDKPWN IINTAALEAV KQER...... ..
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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 RC607 (P16171) (17); nape_entfa, NADH peroxidase from *E. faecalis* (P37062) (12). Indicated in bold type are the redox-active cysteine residues and the Rossman fold Gly-X-Gly-X-X-(Gly/Ala) motifs forming the flavin adenine dinucleotide and NAD(P) binding sites (10, 20).

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 an unusual 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 oxoacid dehydrogenase, and the glycine cleavage system (L-chain). 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 others function 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-

operativity in cofactor binding has been reported for dihydrolipoamide 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.

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REFERENCES

1. **Benen, J. A. E., W. J. H. van Berkel, W. M. A. M. van Dongen, F. Müller, and A. de Kok.** 1989. Molecular cloning and sequence determination of the *lpd* gene encoding lipoamide dehydrogenase from *Pseudomonas fluorescens*. J. Gen. Microbiol. **135:**1787–1797.
2. **Cohen, P. T., and N. O. Kaplan.** 1970. Purification and properties of the pyridine nucleotide transhydrogenase from *Pseudomonas aeruginosa*. J. Biol. Chem. **245:**2825–2836.
3. **Fisher, R. R., and S. R. Earle.** 1982. Membrane-bound pyridine nucleotide transhydrogenases, p. 279–324. *In* J. Everse, B. Anderson, and K.-S. You (ed.), The pyridine nucleotide coenzymes. Academic Press, Inc., New York, N.Y.
4. **Genetics Computer Group.** 1994. Program manual for the Wisconsin package, version 8, September 1994. Genetics Computer Group, Madison, Wis.
5. **Greer, S., and R. N. Perham.** 1986. Glutathione reductase from *Escherichia coli*: cloning and sequence analysis of the gene and relationship to other flavoprotein disulphide oxidoreductases. Biochemistry **25:**2736–2742.
6. **Gustafsson, C., and S. R. Warne.** 1992. Physical map of the *oxyR-trmA* region (minute 89.3) of the *Escherichia coli* chromosome. J. Bacteriol. **174:**7878–7879.
7. **Höjeberg, B., P. Brodelius, J. Rydström, and K. Mosbach.** 1976. Affinity chromatography and binding studies on immobilized adenosine 5′-monophosphate and adenosine 2′,5′-bisphosphate of nicotinamide nucleotide transhydrogenase from *Pseudomonas aeruginosa*. Eur. J. Biochem. **66:**467–475.
8. **Jackson, J. B.** 1991. The proton-translocating nicotinamide adenine dinucleotide transhydrogenase. J. Bioenerg. Biomembr. **23:**715–741.
9. **Louie, D. D., N. O. Kaplan, and J. D. McLean.** 1972. Allosteric effect of 2′-adenylic acid on the *Pseudomonas* pyridine nucleotide transhydrogenase. J. Mol. Biol. **70:**651–664.
10. **McKie, J. H., and K. T. Douglas.** 1991. Evidence for gene duplication forming similar binding folds for NAD(P)H and FAD in pyridine nucleotide-dependent flavoenzymes. FEBS Lett. **279:**5–8.
11. **Poole, L. B., and A. Claiborne.** 1989. The non-flavin redox centre of the streptococcal NADH peroxidase II: evidence for a stabilized cysteine-sulfenic acid. J. Biol. Chem. **264:**12330–12338.
12. **Ross, R. P., and A. Claiborne.** 1991. Cloning, sequence and overexpression of NADH peroxidase from *Streptococcus faecalis* 10C1: structural relationships with the flavoprotein disulfide reductases. J. Mol. Biol. **221:**857–871.
13. **Rydström, J., B. Persson, and E. Carlrenor.** 1987. Transhydrogenases linked to pyridine nucleotides, p. 433–460. *In* D. Dolphin, O. Aramovic, and R. Poulson (ed.), Pyridine nucleotide coenzymes: chemical, biochemical and medical aspects, part B. John Wiley and Sons, New York, N.Y.
14. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
15. **Voordouw, G., S. M. van der Vies, J. K. Eweg, C. Veeger, J. F. L. van Breemen, and E. F. J. van Bruggen.** 1980. Pyridine nucleotide transhydrogenase from *Azotobacter vinelandii*: improved purification, physical properties and subunit arrangement in purified polymers. Eur. J. Biochem. **111:**347–355.
16. **Voordouw, G., S. M. van der Vies, and A. P. N. Themmen.** 1983. Why are two different types of pyridine nucleotide transhydrogenase found in living organisms? Eur. J. Biochem. **131:**527–533.
17. **Wang, Y., M. Moore, H. S. Levinson, S. Silver, C. Walsh, and I. Mahler.** 1989. Nucleotide sequence of a chromosomal mercury resistance determinant from a *Bacillus* sp. with broad-spectrum mercury resistance. J. Bacteriol. **171:**83–92.
18. **Wermuth, B., and N. O. Kaplan.** 1976. Pyridine nucleotide transhydrogenase from *Pseudomonas aeruginosa*: purification by affinity chromatography and physicochemical properties. Arch. Biochem. Biophys. **176:**136–143.
19. **Widmer, F., and N. O. Kaplan.** 1977. *Pseudomonas aeruginosa* transhydrogenase: affinity of substrates for the regulatory site and possible hysteretic behaviour. Biochem. Biophys. Res. Commun. **76:**1287–1292.
20. **Wierenga, R. K., M. C. H. de Maeyer, and W. G. J. Hol.** 1985. Interaction of pyrophosphate moieties with α-helixes in dinucleotide binding proteins. Biochemistry **24:**1346–1357.
21. **Williams, C. H., Jr.** 1992. Lipoamide dehydrogenase, glutathione reductase, thioredoxin reductase and mercuric ion reductase—a family of flavoenzyme transhydrogenases, p. 121–211. *In* F. Müller (ed.), Chemistry and biochemistry of flavoenzymes, vol. III. CRC Press, Boca Raton, Fla.