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Energy-Transducing Nicotinamide Nucleotide Transhydrogenase: Nucleotide Sequences of the Genes and Predicted Amino Acid Sequences of the Subunits of the Enzyme from *Rhodospirillum rubrum*

Mutsuo Yamaguchi[1] and Youssef Hatefi[1]

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Based on the amino acid sequence of the N-terminus of the soluble subunit of the *Rhodospirillum rubrum* nicotinamide nucleotide transhydrogenase, two oligonucleotide primers were synthesized and used to amplify the corresponding DNA segment (110 base pairs) by the polymerase chain reaction. Using this PCR product as a probe, one clone with the insert of 6.4 kbp was isolated from a genomic library of *R. rubrum* and sequenced. This sequence contained three open reading frames, constituting the genes *nntA1*, *nntA2*, and *nntB* of the *R. rubrum* transhydrogenase operon. The polypeptides encoded by these genes were designated α1, α2, and β, respectively, and are considered to be the subunits of the *R. rubrum* transhydrogenase. The predicted amino acid sequence of the α1 subunit (384 residues; molecular weight 40276) has considerable sequence similarity to the α subunit of the *Escherichia coli* and the N-terminal 43-kDa segment of the bovine transhydrogenases. Like the latter, it has a $\beta\alpha\beta$ fold in the corresponding region, and the purified, soluble α1 subunit cross-reacts with antibody to the bovine N-terminal 43-kDa fragment. The predicted amino acid sequence of the β subunit of the *R. rubrum* transhydrogenase (464 residues; molecular weight 47808) has extensive sequence identity with the β subunit of the *E. coli* and the corresponding C-terminal sequence of the bovine transhydrogenases. The chromatophores of *R. rubrum* contain a 48-kDa polypeptide, which cross-reacts with antibody to the C-terminal 20-kDa fragment of the bovine transhydrogenase. The predicted amino acid sequence of the α2 subunit of the *R. rubrum* enzyme (139 residues; molecular weight 14888) has considerable sequence identity in its C-terminal half to the corresponding segments of the bovine and the α subunit of the *E. coli* transhydrogenases.

KEY WORDS: Nicotinamide nucleotide transhydrogenase; subunits; amino acid sequence; *Rhodospirillum rubrum*; proton pump.

INTRODUCTION

The energy-transducing nicotinamide nucleotide transhydrogenases are integral membrane proteins, and catalyze the direct and stereospecific transfer of a hydride ion between the 4A position of NAD(H) and the 4B position of NADP(H) in a reaction that is coupled to transmembrane proton translocation with a H^+/H^- stoichiometry close to unity [equation (1); for recent reviews, see Hatefi and Yamaguchi, 1992; Olausson *et al.*, 1992; Lee and Ernster, 1989].

$$NADH + NADP + H^+_{out} \rightleftharpoons NAD + NADPH + H^+_{in} \quad (1)$$

The bovine mitochondrial transhydrogenase is a homodimer of monomer molecular weight 109,065

[1] Division of Biochemistry, Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California 92037.

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and 1043 amino acid residues (Yamaguchi *et al.*, 1988). The sequence of these residues has been deduced from cDNA clones (Yamaguchi *et al.*, 1988), the enzyme's binding domains for NAD(H) and NADP(H) (Yamaguchi and Hatefi, 1993), and sites of modification by several inhibitors have been determined (Wakabayashi and Hatefi, 1987a,b; Phelps and Hatefi, 1984a,b, 1985), and extensive studies have been done on its membrane topology and mechanism of action (Yamaguchi and Hatefi, 1989, 1991; Yamaguchi *et al.*, 1990; Hatefi and Yamaguchi, 1992). The transhydrogenases from *Escherichia coli* and *Rhodobacter capsulatus* have also been purified (Clarke and Bragg, 1985; Lever *et al.*, 1991). Each enzyme consists of two subunits, with approximate M_r values of 54,000 and 49,000. The *E. coli* enzyme is tetrameric ($\alpha_2\beta_2$) (Hou *et al.*, 1990), and the amino acid sequences of its subunits have been deduced from the genes (Clarke *et al.*, 1986). There is considerable sequence identity between the bovine and the *E. coli* transhydrogenases, especially in the nucleotide binding domains as determined for the bovine enzyme.

In addition to the above, it has long been known that *Rhodospirillum rubrum* chromatophores contain a form of energy-linked nicotinamide nucleotide transhydrogenase of which one subunit is water-soluble and easily removed from the membranes (Fisher and Guillory, 1971). Jackson and coworkers have recently purified this subunit (Cunningham *et al.*, 1992), and shown that its N-terminal 37 residues have considerable sequence identity to that segment of the α subunit of the *E. coli* transhydrogenase and to residues 14–50 of the bovine enzyme. Furthermore, this soluble subunit was shown to be dimeric with a monomer M_r of 43,000, features that are shared with a soluble 43-kDa tryptic fragment of the bovine enzyme.

This paper shows that the *R. rubrum* transhydrogenase message is contained in a cluster of three genes, which have been sequenced and the amino acid sequences of the corresponding subunits deduced therefrom. Comparisons between these data and the amino acid sequences of the bovine and the *E. coli* transhydrogenase will also be presented.

MATERIALS AND METHODS

Materials

Restriction enzymes were obtained from New England Biolabs and Stratagene. pBluescript II KS(+) was from Stratagene. Klenow fragment, Sequenase version 1.0, nucleotide kit for sequencing with 7-deaza-dGTP, and random primed DNA labeling kit were from United States Biochemicals. *Taq*DNA polymerase was from Gibco BRL. BA-85 nitrocellulose filters were from Schleicher & Schuell. [α-^{32}P]dCTP (3000 Ci/mmol) and α-thio-[^{35}S]dATP (1200 Ci/mmol) were from Amersham. NAD-agarose (Type I) was prepared by the method of Mosbach *et al.* (1972).

Amplification of DNA Fragment by PCR

PCR[2] amplification (Saiki *et al.*, 1988) of the portion of the gene encoding the N-terminal sequence of the soluble subunit (α1 subunit) was performed with Twin Block System from Ericomp, Inc. Based on the amino acid sequences 1–7 (MKIAIPK) and 31–37 (FEVIVEQ) of the soluble subunit (α1), degenerate sense and antisense primers containing 288 and 384 different sequences, respectively, were synthesized as shown:

Sense primer

```
5′ ATGAAAATAGCAATACCAAA 3′
     G  T  T  T  T
        C  G  C  G
           C     C
```

Antisense primer

```
3′ AAACTTCAATAACAACTTGT 5′
  G  C  T  T  T  C
        G  G  G
        C     C
```

DNA sequences were amplified with *Taq* DNA polymerase in 100-μl reaction mixtures containing 1 μg of *R. rubrum* DNA, 10 mM Tris-HCl (pH 8.5), 50 mM KCl, 2 mM $MgCl_2$, 0.1% Triton X-100, 250 μM of each dNTP (dATP, dCTP, dGTP, and dCTP), 5 μg of both oligonucleotide primers, and 5 units of *Taq* DNA polymerase. The samples were subjected to 35 amplification cycles, denaturation at 94°C for 90 s, annealing at 46°C for 30 s, and extension from the primers at 72°C for 2 min. The PCR products were separated by electrophoresis on 1.5% agarose gel.

[2] Abbreviations used: PCR, polymerase chain reaction; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; EEDQ, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; FSBA, [(*p*-fluorosulfonyl)benzoyl]-5′-adenosine; PVDF, poly(vinylidene difluoride); bp, base pair; SDS, sodium dodecyl sulfate.

The major amplification product of the predicted size (110 bp) was isolated from the gel and subcloned into the *Eco*RV site of pBluescript II. Five clones inserted by the PCR product were chosen, and their DNA sequences determined as described below. Four of the clones had the same DNA sequence. This sequence was consistent with the amino acid sequence of the N-terminus (residues 1–37) of the soluble subunit of the *R. rubrum* transhydrogenase, as reported by Cunningham *et al.* (1992). The insert of this clone was radiolabeled, using [α^{32}-P]dCTP and random primed DNA labeling kit, and was employed as a probe.

Construction and Screening of the Genomic Library from *R. rubrum*

General cloning techniques were carried out essentially as described by Sambrook *et al.* (1989). Genomic DNA from *R. rubrum* was isolated as described by Saito and Miura (1963). The genomic DNA was digested with *Bam*HI and subjected to agarose gel electrophoresis. The region (5–8 kbp) of the agarose gel that reacted with the PCR product probe was excised, and the DNA fragments were isolated. The genomic library was prepared by ligation of the fragments into the *Bam*HI site of pBluescript II phagemid vector. The library was screened by colony hybridization, using BA-85 nitrocellulose filters. Replicate filters were prehybridized at 42°C for 2 h in a solution consisting of 50% formamide, 5 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.2), 1 × Denhardt's solution (Denhardt, 1966), 0.2% SDS, and calf thymus DNA (100 μg/ml). Hybridization was carried out overnight at 42°C in prehybridization solution with the [α-^{32}P]dCTP-labeled probes (~10^6 cpm/ml). The filters were washed at 42°C for 1 h in 5 × SSC containing 50% formamide and 0.2% SDS, and then in 5 × SSC containing 0.2% SDS. They were further washed in 2 × SSC at 42°C for 30 min. Autoradiography was carried out overnight with Fuji RX film and intensifying screens.

DNA Sequencing Strategy

The DNA insert in the phagemid was cut with restriction enzymes and the fragment ends were blunted by fill-in with Klenow fragment and dNTPs. Each fragment was subcloned into the *Eco*RV site of pBluescript II. After purification and alkali denaturation (Chen and Seeburg, 1985) of phagemid, DNA sequencing was carried out by the dideoxynucleotide method (Sanger *et al.*, 1977) with Sequenase and 7-deaza-dGTP sequencing kit. Universal primers, M13-20 primer and reverse primer, and internal unique primers 18 bases in length were used for DNA sequencing.

Analysis of Nucleotide and Protein Sequences

The University of Wisconsin Genetic Computer Group's software programs were used to analyze the sequence data (Devereux *et al.*, 1984). Comparison of the polypeptides was made with the BESTFIT and PILEUP programs.

Purification of the Soluble Subunit of *R. rubrum* Transhydrogenase

R. rubrum cells were grown photosynthetically (Ormerod *et al.*, 1961), and crude soluble subunit

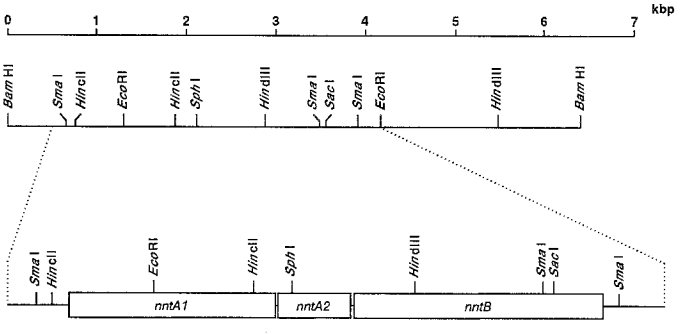
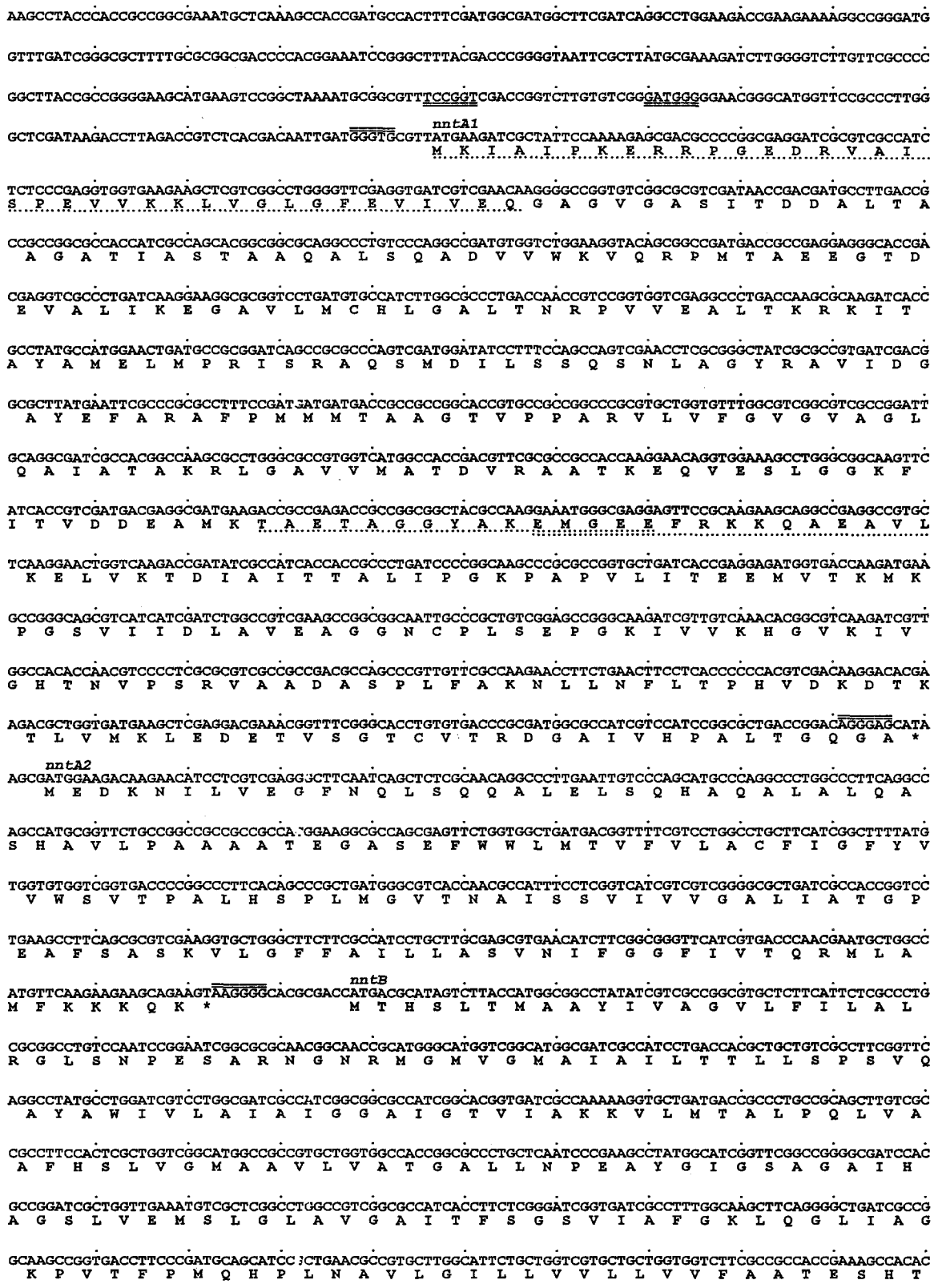


Fig 1. Restriction map of the insert of the isolated clone pRRTH-11 and its expanded transhydrogenase coding region. Three structural genes (*nntA1*, *nntA2*, and *nntB*) of *R. rubrum* transhydrogenase are encoded in the expanded region of the insert (6.4 kbp *Bam*HI fragment) of pRRTH-11. Structural genes *nntA1*, *nnt2*, and *nntB* encode subunits α1, α2, and β, respectively. Both strands of the expanded *Sma*I-*Eco*RI 3.7 kbp region were completely sequenced.


nntA1
nntA2
nntB

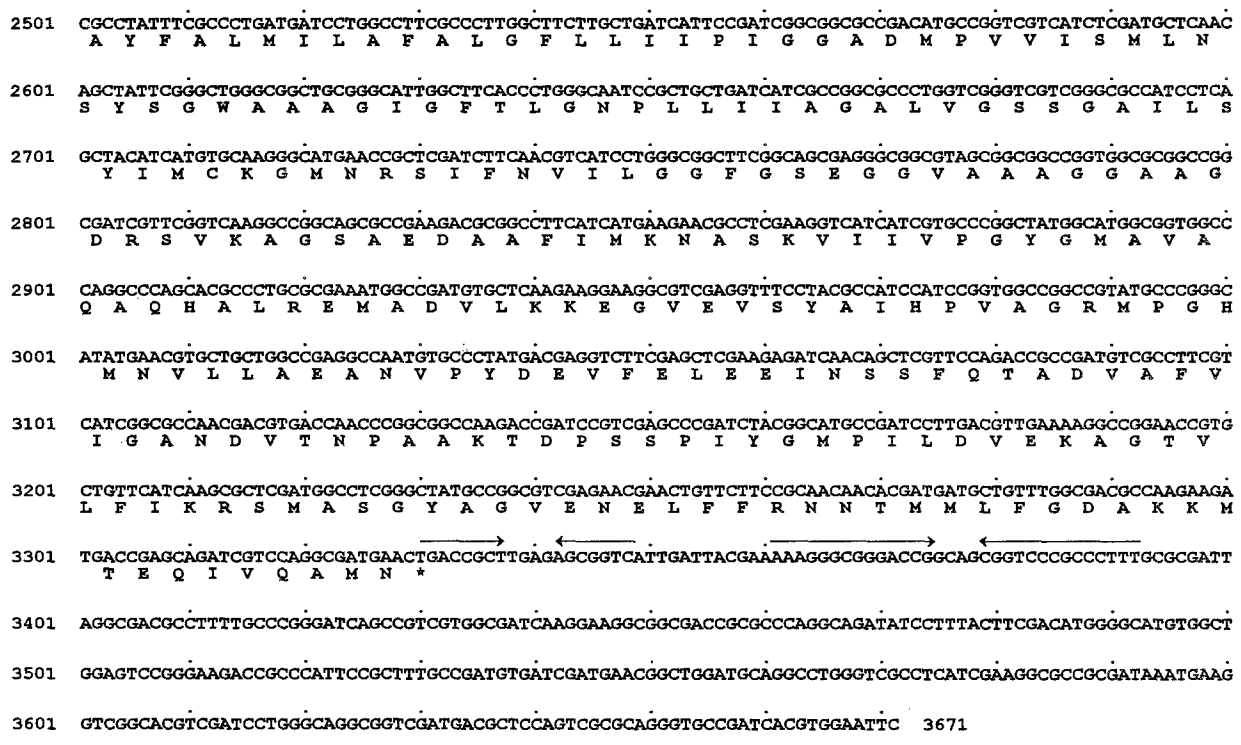


Fig. 2. Nucleotide sequences of *R. rubrum* transhydrogenase genes and the predicted amino acid sequences of the subunits. The amino acid sequences determined by protein analysis (Cunningham *et al.*, 1992; Palmer *et al.*, 1993) are underscored by dotted lines. The putative Shine–Dalgarno sequences are doubly overlined. The sequences similar to the tentative promoter sequences (−10, −35 regions) of α-purple bacteria (Steinrücke and Ludwig, 1993) are doubly underlined. Possible stem/loops are indicated by converging arrows. The GenBank Accession Number for the above sequences is U01158.

(α1) and chromatophores were prepared from 12-g batches of cells as reported by Fisher and Guillory (1971). The crude soluble subunit (α1) (20 ml) was desalted by passing through Sephadex G-50 column (2.5 × 30 cm) equilibrated with 10 mM Tris-HCl (pH 7.4) containing 0.4 mM dithiothreitol (Buffer A) and loaded onto a NAD-agarose column (2.5 × 10 cm) equilibrated with Buffer A. The column was washed with 70 ml each of Buffer A containing 20 mM NaCl and then with Buffer A. The soluble subunit (α1) was eluted with 50 ml of Buffer A containing 0.5 mM NADH and then with 50 ml of Buffer A. Fractions (60 ml) that exhibited transhydrogenase activity in the presence of washed membranes were loaded onto a DEAE-Sephadex A-50 column (1 × 13 cm) equilibrated with 10 mM Tris-HCl (pH 8.0) containing 0.1 mM NADP, 14 mM 2-mercaptoethanol, and 1 mM dithiothreitol. The soluble subunit (α1) was eluted with a linear gradient (0 to 20 mM) of ammonium sulfate in the same buffer. Active fractions (11 ml) were concentrated to ~500 μl using Centricon-30 concentrator.

Detection of Peptides by Immunoblotting

Protein samples were subjected to 13% SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). Peptides were transferred onto PVDF membranes using a Bio-Rad Mini Trans-Blot apparatus. Immunoblotting was carried out using affinity-purified antibodies raised to the N-terminal 43-kDa peptide and the C-terminal 20-kDa peptide of bovine transhydrogenase as reported previously (Yamaguchi and Hatefi, 1991).

RESULTS AND DISCUSSION

Cloning of the Genes of Transhydrogenase Subunits

Four PCR clones were isolated whose insert sequences were consistent with the amino acid sequences of the N-terminus (residues 1–37) of the soluble *R. rubrum* transhydrogenase subunit (α1). The insert (110 bp) of one clone was radiolabeled and employed as a probe. When Southern blotting

was carried out on the *R. rubrum* genomic DNA, one 6.4 kbp *Bam*HI fragment strongly reacted with this probe. Therefore, 5–8-kbp fragments were isolated from the *Bam*HI digest of *R. rubrum* genomic DNA and a genomic library was constructed by ligation of the fragments into the *Bam*HI site of pBluescript II. The library was screened by colony hybridization. From a population of 3000 colonies one clone strongly reacted with the probe. This clone (pRRTH-11) was found to contain the 6.4-kbp insert, which was subjected to further restriction and sequence analyses.

DNA Sequence Analysis

Figure 1 displays the restriction map of the 6.4-kbp *Bam*HI fragment of pRRTH-11. The PCR probe reacted with a 1.3-kbp *Bam*HI-*Eco*RI fragment, but not with the initial 750 bp *Bam*HI-*Hinc*II portion of this fragment. Therefore, the sequence encoding the N-terminus of the soluble subunit (α1) was located in the following 550 bp *Hinc*II-*Eco*RI region. Sequencing of this region revealed that the gene encoding the soluble subunit (α1) starts from 850 bp downstream of the first *Bam*HI site and that the gene is coded toward the second *Bam*HI site. Furthermore, the 3.7-kbp *Sma*I-*Eco*RI region (see Fig. 1) was found to have three open reading frames. These genes, whose nucleotide sequences are shown in Fig. 2, were designated *nntA1*, *nntA2*, and *nntB* (*nnt* for nicotinamide nucleotide transhydrogenase), and the polypeptides they encode were designated α1, α2, and β, respectively. The putative Shine–Dalgarno sequence (Shine and Dalgarno, 1975) for ribosome binding is found 5–10 base pairs upstream from the initiation codon of each gene, as shown in Fig. 2. The inverted repeats in the region following the *nntB* gene can form step/loop structures and are considered to be the terminator of the *nnt* operon.

It is known that *R. rubrum* has a high genomic GC content (60–70%) (Osawa *et al.*, 1990). The GC contents of *nntA1*, *nntA2*, and *nntB* are 65.5, 61.2, and 64.1%, respectively. The amino acid compositions of the three subunits deduced from the gene sequences are shown in Table I. *nntA1*, *nntA2*, and *nntB* code for 3 polypeptides with 384, 139, and 464 amino acid residues and calculated molecular weights of 40276, 14888, and 47808, respectively. The polarity indices of these polypeptides (α1, α2, and β) calculated by summation of the molar percentage of the polar amino acid residues (D, N, E, Q, S, T, H, K, and R) (Capaldi and Vanderkooi, 1972) are 40, 33, and 29%, respectively. The polarity index (29%) of the latter polypeptide is much smaller than that (33%) of the corresponding *E. coli* β subunit.

Table I. Amino Acid Compositions of the Transhydrogenase Subunits from *Rhodospirillum rubrum*

	Residues/subunit		
Amino acid	α1	α2	β
Asp	17	1	10
Asn	6	4	19
Thr	30	6	18
Ser	15	11	30
Glu	27	6	18
Gln	10	8	9
Pro	19	4	18
Gly	33	9	52
Ala	55	21	71
Cys	3	1	1
Val	42	14	43
Met	15	5	25
Ile	22	8	37
Leu	29	16	48
Tyr	4	1	11
Phe	8	11	20
Lys	27	6	15
His	5	3	8
Arg	16	1	9
Trp	1	3	2
Total	384	139	464

The Polypeptide Encoded by *nntA1*, *nntA2*, and *nntB*

The amino acid sequences of the polypeptides encoded by *nntA1*, *nntA2*, and *nntB* are aligned in Fig. 3 with the amino acid sequence of the bovine (single subunit) and *E. coli* (two subunits) transhydrogenases. Also shown in Fig. 3 is the predicted amino acid sequence of a putative protein encoded by an *Eimeria tenella* gene.

The presumption that *nntA1*, *nntA2*, and *nntB* encode three subunits (respectively α1, α2, and β) of the *R. rubrum* nicotinamide nucleotide transhydrogenase is based on the following considerations: (a) The soluble 40-kDa polypeptide (α1) is required for the expression of transhydrogenase activity by washed *R. rubrum* chromatophores. This polypeptide cross-reacts with antibody to the N-terminal 43-kDa tryptic fragment of the bovine transhydrogenase (Fig. 4, panel A), and, like the latter, it is dimeric

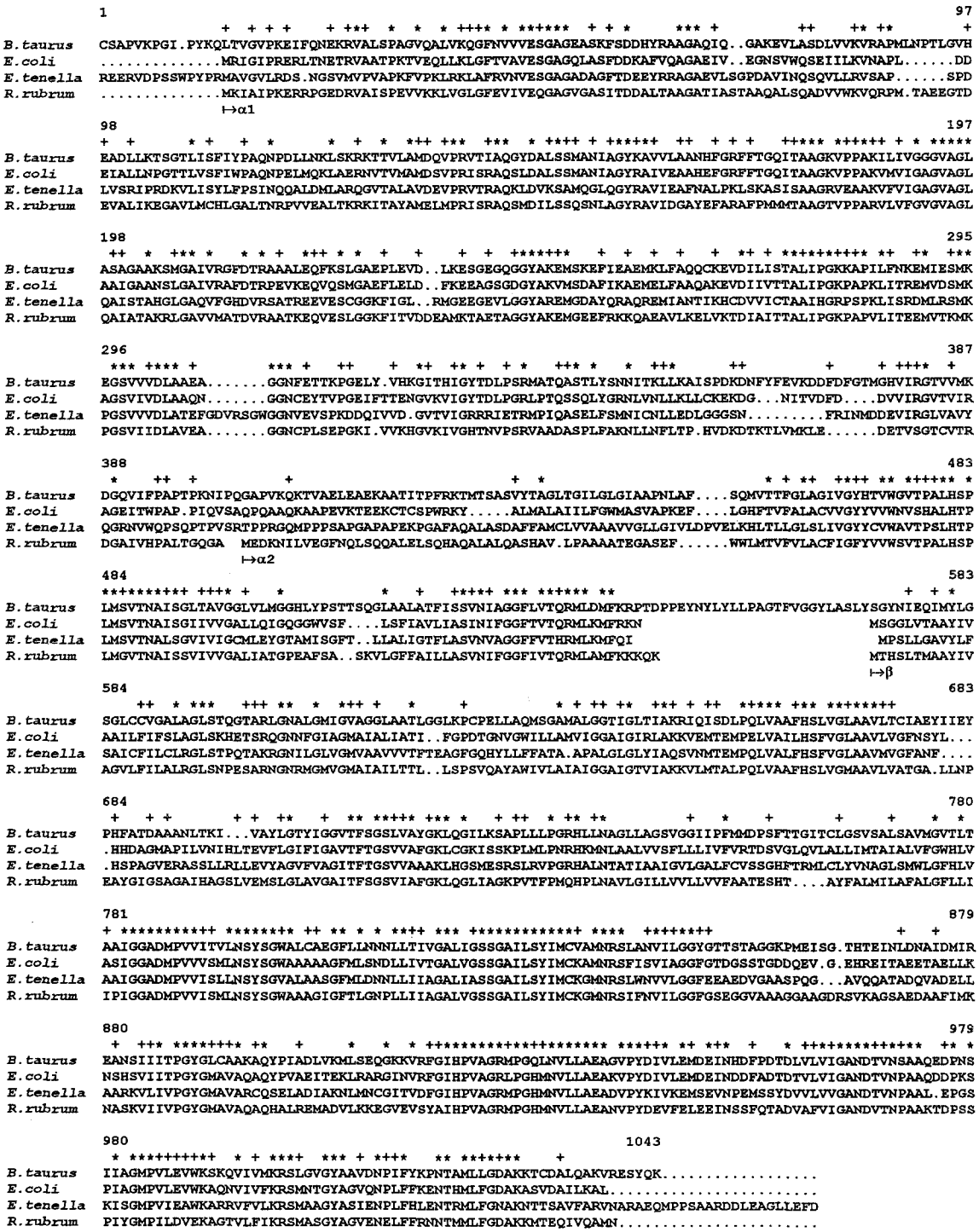


Fig. 3. Alignment of the amino acid sequences of transhydrogenases from bovine mitochondria (one subunit), *Escherichia coli* (two subunits), *Eimeria tenella* (one subunit, see text), and *Rhodospirillum rubrum* (three subunits). The presumed amino acid sequence of the *E. tennella* enzyme has been cut into two halves. Its N-terminal half is aligned with the C-terminal half of the bovine enzyme, and its C-terminal half is aligned with the N-terminal half of the bovine enzyme. Identities among all sequences are shown by asterisks, and among any three sequences, by plus signs. Alignment gaps are shown by dots. The sequence of each peptide was deduced from the nucleotide sequence of the genes or cDNA.

(Cunningham *et al.*, 1992) and binds to NAD agarose (McFadden and Fisher, 1978). The amino acid sequence of $\alpha 1$ shows considerable sequence identity to the α subunit of the *E. coli* and the N-terminal hydrophilic domain of the bovine transhydrogenases. (b) Membrane extracts of *R. rubrum* chromatophores, which in the presence of $\alpha 1$ exhibit transhydrogenase activity, contain a 48-kDa polypeptide which cross-reacts with antibody to the C-terminal 20-kDa fragment of the bovine transhydrogenase (Fig. 4, panel B). The 48-kDa polypeptide encoded by *nntB* has extensive sequence identity to the β subunit of the *E. coli* and the C-terminal half of the bovine transhydrogenases (Fig. 3). (c) The 15-kDa polypeptide encoded by *nntA2* has yet to be identified as a membrane-bound component of *R. rubrum* chromatophores. However, the amino acid sequence of the C-terminal half of this polypeptide has considerable sequence identity to the corresponding segments of the bovine and the α subunit of the *E. coli* transhydrogenases. (d) As seen in the alignment of Fig. 5, the hydropathy plots of the *R. rubrum* $\alpha 1$, $\alpha 2$, and β polypeptides are highly analogous to those of the α and β subunits of the *E. coli* transhydrogenase. Therefore, it seems reasonable to assume that the *R. rubrum* operon containing the genes *nntA1*, *nntA2*, and *nntB* encodes a three-subunit nicotinamide nucleotide transhydrogenase, of which subunit $\alpha 1$ is a peripheral, water-soluble protein and subunits $\alpha 2$ and β are integral membrane proteins.

Figure 3 also includes the amino acid sequence of a putative protein (single polypeptide) as predicted from the cDNA of *Eimeria tenella* (Kramer *et al.*, 1993; Vermeulen *et al.*, 1993), which is an intracellular protozoan parasite of the cecal epithelium of chickens. Whether this gene encodes a viable transhydrogenase is not yet known. However, the amino acid sequence predicted therefrom bears considerable similarity to those of the other three transhydrogenases of Fig. 3, except for the following interesting difference. The N-terminus of the *E. tenella* putative protein starts at a position corresponding in Fig. 3 to residue 573 of the bovine enzyme (the *E. coli* and *R. rubrum* β subunits start at a position corresponding in Fig. 3 to residue 572 of the bovine transhydrogenase). Thus, the N-terminus of the *E. tenella* putative protein has a 300-residue-long hydrophobic stretch. This is followed by a 600-residue-long hydrophilic stretch, corresponding to the NADP(H) followed by the NAD(H) binding domains of the bovine transhydrogenase. The *E. tenella* putative protein ends with a 130-residue-long hydrophobic stretch, which corresponds to the $\alpha 2$ subunit of the *R. rubrum* and the C-terminal hydrophobic domain of the α subunit of the *E. coli* transhydrogenases. Therefore, in examining Fig. 3, the following points should be considered. (i) The predicted amino acid sequence of the *E. tenella* putative protein has been divided into two halves. Its N-terminal half has been aligned with the C-terminal half of the bovine transhydrogenase, and its C-terminal half with the N-terminal half of the bovine enzyme. (ii) All alignments are with respect to the amino acid sequence of the bovine transhydrogenase, whose amino acid sequence numbers are

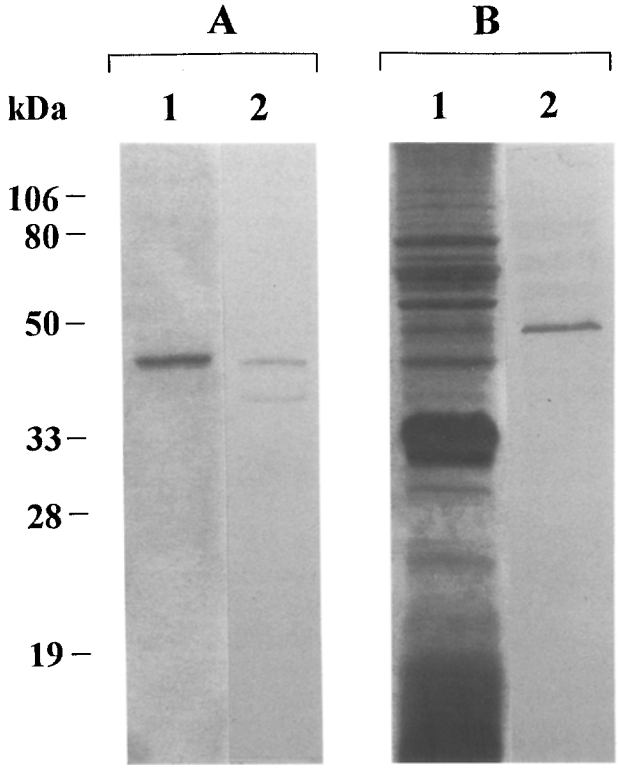


Fig. 4. Immunoblotting of transhydrogenase subunits. The purified soluble subunit ($\alpha 1$) (0.3 μg/lane) and deoxycholate extracts of KCl-washed *R. rubrum* chromatophores (2.4 μg/lane) were subjected to SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. Immunoblotting was carried out using affinity-purified antibodies as described previously (Yamaguchi and Hatefi, 1991). Panel A, soluble subunit ($\alpha 1$): lane 1, protein staining with Coomassie blue; lane 2, immunostaining with antibody to the N-terminal 43-kDa peptide of bovine transhydrogenase. The lower M_r band in this lane is probably a degradation product of $\alpha 1$. Panel B, chromatophore extract: lane 1, staining with Coomassie blue; lane 2, immunostaining with antibody to the C-terminal 20-kDa peptide of bovine transhydrogenase. As shown by the arrowhead, a 48-kDa peptide was immunostained with antibody to the bovine C-terminal 20-kDa peptide.

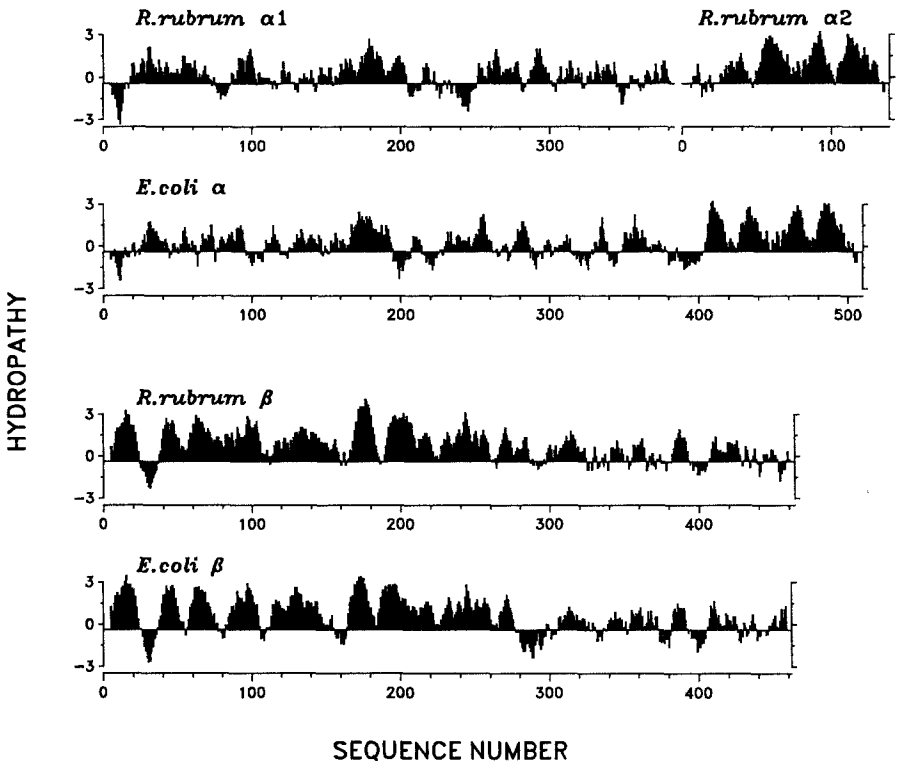


Fig. 5. Hydropathy profiles of the *E. coli* and *R. rubrum* transhydrogenases. Hydropathy scores were calculated by the method of Kyte and Doolittle (1982), using a setting of nine residues. The horizontal line at −0.4 on the ordinate denotes the average hydropathy of 84 fully sequenced soluble proteins. The areas above and below this line indicate relative hydrophobic and hydrophilic regions, respectively.

indicated at the start and the end of each line. (iii) The N-termini of the α1, α2, and β subunits of the *R. rubrum* enzyme have been so labeled. (iv) Residue identities among the four sequences are marked by asterisks, and among any three sequences by plus signs.

In addition to the sequence similarities seen in Fig. 3 among the bovine, *E. coli*, and the *R. rubrum* transhydrogenases, there are several interesting features of the amino acid sequence of the latter that are worthy of note.

1. The NAD(H) binding domain of the bovine enzyme is marked by a βαβ fold followed by an EEDQ-modifiable and NADH(and NMNH)-protectable Glu_{232}, a FSBA-modifiable and NADH-protectable Tyr_{245}, and a DCCD-modifiable and NADH(and AMP)-protectable Glu_{257} (Fig. 6). In the corresponding region of the α1 subunit of the *R. rubrum* transhydrogenase, there is also a βαβ fold (with the highest score of 11, see Wierenga *et al.*, 1986), and a conserved Tyr corresponding to Tyr_{245} of the bovine enzyme (Fig. 6). The glutamic acid residues modified by EEDQ and DCCD in the bovine enzyme are also present in the corresponding positions of the α subunit of the *E. coli*, but not in the α1 subunit of the *R. rubrum* protein (Figs. 3 and 6).

2. In the NADP(H) binding domain of the bovine transhydrogenase, there is an EEDQ-modifiable and NMNH-protectable Glu_{880} and a FSBA-modifiable Tyr_{1006}. The latter, but not the former, is conserved in the corresponding position of the β subunit of the *R. rubrum* enzyme (Fig. 6).

3. According to the site-directed mutagenesis studies of Olausson *et al.* (1992), the *E. coli* tyrosine residues corresponding to the FSBA-modifiable Tyr_{245} and Tyr_{1006} of the bovine enzymes are not essential residues. It is interesting, however, that these tyrosine residues are conserved in all the four sequences shown in Fig. 3.

4. A striking feature of Fig. 3 is the high degree of sequence identity among the four proteins listed downstream of the bovine residue 781. This region

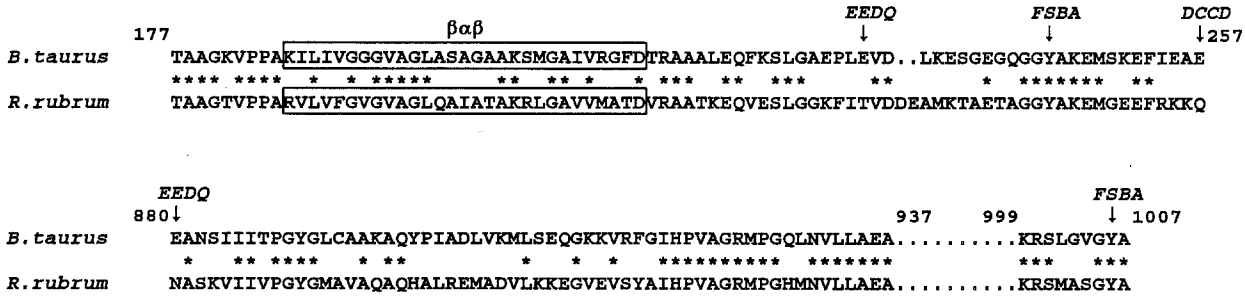


Fig. 6. Portions of the NAD(H)-binding (residues 177–257) and NADP(H)-binding (residues 880–1007) domains of the bovine transhydrogenase and the corresponding regions of the α1 and β subunits, respectively, of the *R. rubrum* transhydrogenase. Substrate-protectable residues modified by EEDQ, FSBA, and DCCD in the bovine enzyme are marked by arrows, and $\beta\alpha\beta$ folds, which are hallmarks of protein binding domains for ADP, ATP, NAD, and FAD (Wierenga *et al.*, 1986), are boxed.

starts with a short hydrophobic segment, corresponding to two possible membrane-spanning α helices, followed by a 200-residue-long hydrophilic segment, which we have shown in the bovine enzyme to include the NADP(H) binding domain. The high degree of sequence conservation in this region may be relevant to the mechanism of energy transduction by the transhydrogenase, as we have proposed elsewhere (Yamaguchi and Hatefi, 1989; Yamaguchi *et al.*, 1990; Hatefi and Yamaguchi, 1992). As seen in equation (1) in the reverse direction, oxidation of NADPH by NAD results in uphill proton translocation and establishment of a transmembrane proton electrochemical potential. Since the NADPH/NADP and the NADH/NAD couples have nearly the same reduction potentials ($\Delta E_{m,7} \simeq 5$ mV) and all substrates and products are on the same side of the membrane, the only source of energy for uphill proton translocation in the reversal of equation (1) is the difference in the concentrations (or binding energies) of reactants (NADPH and NAD) and products (NADP and NADH). Furthermore, since the scalar transhydrogenation reaction [equation (1)] does not involve release or uptake of protons (hydride ion transfer between NAD and NADP is direct), and the enzyme does not contain a prototropic cofactor capable of translocating protons across the membrane, it follows that proton uptake and release across the membrane must be accomplished by pK_a changes of appropriate amino acid residues of the enzyme itself. These considerations suggested, therefore, that energy transduction by the transhydrogenase occurs via substrate-induced conformation change of the protein. In this manner, the difference in the binding energies of substrates and products would be communicated to the protein, resulting in pK_a changes of appropriate residues and proton uptake and release on opposite sides of the membrane. Consistent with this view, we have shown that NADP and NADPH (but not NAD or NADH) binding causes different changes in the conformation of the bovine enzyme (Yamaguchi and Hatefi, 1989; Yamaguchi *et al.*, 1990), and changes the pK_a of Cys_{893}: 9.5 in the presence of NADP and 8.7 in the presence of NADPH (Yamaguchi and Hatefi, 1989). It is, therefore, noteworthy that the NADP(H) binding domain of the transhydrogenase and a nearby hydrophobic segment capable of membrane intercalation are highly conserved in the four sequences shown in Fig. 3.

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