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

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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 $\alpha 1$, $\alpha 2$, and β , respectively, and are considered to be the subunits of the *R*. rubrum transhydrogenase. The predicted amino acid sequence of the $\alpha 1$ 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 α 1 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 $\alpha 2$ subunit of the R. rubrum enzyme (139 residues; molecular weight 14888) has considerable sequence identity in its Cterminal 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].

$$ADH + NADP + H_{out}^+$$

$$\Rightarrow$$
 NAD + NADPH + H⁺_{in} (1)

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

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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 -³²P]dCTP (3000 Ci/mmol) and α -thio-[³⁵S]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

PCR² amplification (Saiki *et al.*, 1988) of the portion of the gene encoding the N-terminal sequence of the soluble subunit (α 1 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 (α 1), degenerate sense and antisense primers containing 288 and 384 different sequences, respectively, were synthesized as shown:

Sense primer							
5' ATGAAAATAGCAATACCAAA 3'							
	G	Т	Т	Т	Т		
		С	G	С	G		
			С		С		
Antisense primer							
3' AAACT	ГТСА	ATA	ACA	ACT	TGT 5'		
G	С	Т	Т	Т	С		
		G	G	G			
		C		C			

DNA sequences were amplified with Taq DNA polymerase in 100- μ l reaction mixtures containing 1 μ g of *R. rubrum* DNA, 10 mM Tris-HCl (pH 8.5), 50 mM KCl, 2 mM MgCl₂, 0.1% Triton X-100, 250 μ M of each dNTP (dATP, dCTP, dGTP, and dCTP), 5 μ g 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)-2ethoxy-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 $[\alpha^{32}$ -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 BamHI 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 BamHI 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 2h in a solution consisting of 50% formamide, $5 \times SSC$ $(1 \times SSC = 0.15 \text{ M NaCl}, 0.015 \text{ M} \text{ sodium citrate},$ pH 7.2), $1 \times$ Denhardt's solution (Denhardt, 1966), 0.2% SDS, and calf thymus DNA (100 μ g/ml). Hybridization was carried out overnight at 42°C in

prehybridization solution with the $[\alpha^{-32}P]dCTP$ labeled probes (~10⁶ 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 7deaza-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 α 1, α 2, and β , respectively. Both strands of the expanded *SmaI-Eco*RI 3.7 kbp region were completely sequenced.

1	AAGCCTACCCACCGCCGGCGAAATGCTCAAAGCCACCGATGCCACTTTCGATGGCGATGGCTTCGATCAGGCCTGGAAGACCGAAGAAAAGGCCCGGGATG
101	GTTTGATCGGGCGCTTTTGCGCGGCGACCCCCCGGGAAATCCGGGGCTTTACGACCCGGGGTAATTCGCTTATGCGAAAGATCTTGGGGTCTTGTTCGCCCC
201	GGCTTACCGCCGGGGAAGCATGAAGTCCGGCTAAAATGCGGCGTT <u>TCCGGT</u> CGACCGGTCTTGTGTCCG <u>GATGGG</u> GGAACGGGCATGGTTCCGCCCTTGG
301	nntA1 GCTCGATAAGACCTTAGACCGTCTCACGACAATTGATGGGTGCGTTATGAAGATCGCTATTCCAAAAGAGCGACGCCCCGGCGAGGATCGCGTCGCCATC M.K.I.A.I.P.K.E.R.R.P.G.E.D.R.V.A.I.
401	TCTCCCGAGGTGGTGAAGAAGCTCGTCGGCCTGGGGTTCGAGGTGATCGTCGAACAAGGGGCCGGTGTCGGCGCGCGTGAAACCGACGACGACGACGACGACGACGACGACGACGACG
501	CCGCCGGCGCCACCATCGCCAGGCGCGGCGCGCGGGGCCCTGTCCCAGGCGATGTGGTCTGGAAGGTACAGCGGCGATGACCGCCGAGGAGGGCACCGA A G A T I A S T A A Q A L S Q A D V V W K V Q R P M T A E E G T D
601	CGAGGTCGCCTGATCAAGGAAGGCGCGGTCCTGATGTGCCATCTTGGCGCCCTGACCAACCGTCCGGTGGTCGAGGCCCTGACCAAGCGCAAGATCACC E V A L I K E G A V L M C H L G A L T N R P V V E A L T K R K I T
701	GCCTATGCCATGGAACTGATGCCGCGGGGCGAGCGCGCGC
801	GCGCTTATGAATTCGCCCGCGCCTTTCCGATJATGATGACGCCGCCGCCGCGCGCGCGCGCGCGCGCGCGCG
901	GCAGGCGATCGCCACGGCCAAGCGCCCTGGGCGCCGTGGTCATGGCCACCGACGTCGCGCCACGCAAGGAACAGGTGGAAAGCCTGGGCGGCAAGTTC Q A I A T A K R L G A V V M A T D V R A A T K E Q V E S L G G K F
1001	ATCACCGTCGATGACGAGGCGATGAAGACCGCCGAGACCGCCGCGGCGGCGAGGAATGGGCCGAGGAGTTCCGCAAGAAGCAGGCCGAGGCCGTGC I T V D D E A M K T A E T A G G Y A K E M G E E F R K K Q A E A V L
1101	TCAAGGAACTGGTCAAGACCGATATCGCCATCACCACCGCCTGATCCCCGGCGAGCCGGTGCTGATCACCGAGGAGATGGTGACCAAGATGAA K E L V K T D I A I T T A L I P G K P A P V L I T E E M V T K M K
1201	GCCGGGCAGCGTCATCATCGATCTGGCCGTCGAAGCCGGCGGCAATTGCCCGGCGGGCAAGATCGTTGTCAAACACGGCGTCAAGATCGTT P G S V I I D L A V E A G G N C P L S E P G K I V V K H G V K I V
1301	GGCCACACCÀACGTCCCCTCGCGCGGCGGCGGCGGCGGCGGCGGCGGCGGC
1401	AGACGCTGGTGATGAAGCTCGAGGACGAAACGGTTTCGGGCACCTGTGTGACCCGCGCATCGCCCATCGTCCATCCGGCGCTGACCGGAC $\overline{\lambda}\overline{G}\overline{G}\overline{G}\overline{G}\overline{G}\overline{G}\overline{G}\overline{G}\overline{G}G$
1501	nnta2 AGCGATGGAAGACAAGAACATCCTCGTCGAGGGCCTTCAATCAGCTCTCGCAACAGGCCCTTGAATTGTCCCAGCATGCCCAGGCCCTGGCCCTTCAGGCC M E D K N I L V E G F N Q L S Q Q A L E L S Q H A Q A L A L Q A
1601	AGCCATGCGGTTCTGCCGGCCGCCGCCGCCGCCGCGGAGGCCGCCGGCGGGGGG
1701	TGGTGTGGTGGTGGCGCGCGGCGCGCGCGGCGCGCGGGGGG
1801	TGAAGCCTTCAGCGCGTCGAAGGTGCTGGGCTTCTTCGCCATCCTGCGAGCGTGAACATCTTCGGCGGGTTCATCGTGACCCAACGAATGCTGGCC E A F S A S K V L G F F A I L L A S V N I F G G F I V T Q R M L A
1901	nntB ATGTTCAAGÀAGAAGCAGAÁGT <mark>ÀAGGGG</mark> CÀCGCGACCATGACGCATAGTCTTACCATGGCGGCCTATATCGTCGCCGGCĠTGCTCTTCATTCTCGCCCTĠ M F K K K Q K * M T H S L T M A A Y I V A G V L F I L A L
2001	CGCGGCCTGTCCAATCCGGAATCGGCGCAACGGCAACGGCATGGGGCATGGCGATCGCCATCCTGACCACGCTGCGCTGCGCCTTCGGTTC R G L S N P E S A R N G N R M G M V G M A I A I L T T L L S P S V Q
2101	AGGCCTATGCCTGGATCGTCTGGCGATCGCCATCGGCGCGCCATCGGCGCGAGATGACGCCGGAGGTGGTGATGACCGCCGCGCGCG
2201	CGCCTTCCACTCGCTGGTCGGCATGGCCGCCGTGCTGGTCGGCCGCGCGCG
2301	GCCGGATCGCTGGTTGAAATGTCGCTCGGCCTCGGCCGTCGGCGCCATCACCTTCCGGGATCGCCTTTGGCAAGCTTCAGGGGCTGATCGCCG A G S L V E M S L G L A V G A I T F S G S V I A F G K L Q G L I A G
2401	GCAAGCCGGTGACCTTCCCGATGCAGCATCC CTGAACGCCGTGCTGCTGGTGGTCGTGGTGGTCTTCGCCGCCACCGAAAGCCACAC K P V T F P M Q H P L N A V L G I L L V V L L V V F A A T E S H T

2501	CGCCTATTICGCCCTGATGATCCTGGCCTTCGCCCTTGGCTGTCGTCGATCGTCGATCGGCGGCGCGACATGCCGGTCGTCATCTCGATGCTCAAC A Y F A L M I L A F A L G F L L I I P I G G A D M P V V I S M L N
2601	AGCTATTCGGGCTGGGGCGGCTGCGGGCATTGGCTTCACCCTGGCGCATCCGCGCTGGTCGGCGCCCTGGTCGGGCGCCGCCGCCATCCTCA S Y S G W A A A G I G F T L G N P L L I I A G A L V G S S G A I L S
2701	GCTACATCATGTGCAAGGGCATGAACCGCTCGATCTTCAACGTCATCCTGGGCGGCGCGCGGGGGGGG
2801	CGATCGTTCGGTCAAGGCCGGCGCGCGCGGCGCGGCCTTCATCATGAAGAACGCCTCGAAGGTCATCATCGTGCCCGGCTATGGCATGGCGGTGGCC D R S V K A G S A E D A A F I M K N A S K V I I V P G Y G M A V A
2901	CAGGCCCAGCACGCCCTGCGCGAAATGGCCGATGTGCTCCAAGAAGGAAG
3001	ATATGAACGIGCTGCTGGCCGAGGCCAAIGTGCCCTATGACGAGGTCTTCGAGCTCGAAGAGATCAACAGCTCGTTCCAGACCGCCGAIGTCGCCTTCGT M N V L L A E A N V P Y D E V F E L E E I N S S F Q T A D V A F V
3101	CATCGGCGCCAACGACGTGACCAACCCGGCGGGCCAAGACCGATCCGTCGAGCCCGATCTTACGGCATGCCGATCCTTGACGTTGAAAAGGCCGGAACCGTG I G A N D V T N P A A K T D P S S P I Y G M P I L D V E K A G T V
3201	CTGTTCATCAAGCGCTCGATGGCCTCGGGCTATGCCGGCGTCGAGAACGAAC
3301	TGACCGAGCAGATCGTCCAGGCGATGAACTGACCGCTTGAGAGCGGTCATTGATTACGAAAAAGGGCGGGACCGGCAGCGGTCCCGCCCTTTGCGCGATT T E Q I V Q A M N *
3401	AGGCGACGCCTTTTGCCCGGGATCAGCCGTCGTGGCGATCAAGGAAGG
3501	GGAGTCCGGGAAGACCGCCCATTCCGCTTTGCCGATGTGATCGATGAACGGCTGGATGCAGGCCTGGGTCGCCTCATCGAAGGCGCCGCGATAAATGAAG
3601	GTCGGCACGTCGATCCTGGGCAGGCGGTCGATGACGCTCCAGTCGCGCAGGGTGCCGATCACGTGGAATTC 3671

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.

(α 1) and chromatophores were prepared from 12-g batches of cells as reported by Fisher and Guillory (1971). The crude soluble subunit (α 1) (20 ml) was desalted by passing through Sephadex G-50 column $(2.5 \times 30 \text{ cm})$ equilibrated with 10 mM Tris-HCl (pH 7.4) containing 0.4 mM dithiothreitol (Buffer A) and loaded onto a NAD-agarose column $(2.5 \times 10 \text{ cm})$. 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 (α 1) 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 $(1 \times 13 \text{ cm})$ 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 (α 1) was eluted with a linear gradient (0 to 20 mM) of ammonium sulfate in the same buffer. Active fractions (11 ml) were concentrated to \sim 500 μ l using Centricon-30 concentrator.

Detection of Peptides by Immunoblotting

Protein samples were subjected to 13% SDSpolyacrylamide gel electrophoresis (Laemmli, 1970). Peptides were transferred onto PVDF membranes using a Bio-Rad Mini Trans-Blot apparatus. Immunoblotting was carried out using affinitypurified 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 (α 1). 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 BamHI fragment of pRRTH-11. The PCR probe reacted with a 1.3-kbp BamHI-EcoRI fragment, but not with the initial 750 bp BamHI-HincII portion of this fragment. Therefore, the sequence encoding the N-terminus of the soluble subunit (α 1) was located in the following 550 bp HincII-EcoRI region. Sequencing of this region revealed that the gene encoding the soluble subunit (α 1) starts from 850 bp downstream of the first BamHI site and that the gene is coded toward the second BamHI site. Furthermore, the 3.7-kbp SmaI-EcoRI 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 $\alpha 1$, $\alpha 2$, 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 ($\alpha 1$, $\alpha 2$, and β) calculated by summation of the molar

	Residues/subunit			
Amino acid	αΙ	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	

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

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 sub-unit.

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 nntBencode three subunits (respectively $\alpha 1$, $\alpha 2$, and β) of the *R. rubrum* nicotinamide nucleotide transhydrogenase is based on the following considerations: (a) The soluble 40-kDa polypeptide ($\alpha 1$) is required for the expression of transhydrogenase activity by washed *R. rubrum* chromatophores. This polypeptide crossreacts 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

	1	97
	+ + + + + +*+ * * * +++* * **+*** + + * *** *** ++ ++	+
B.taurus	CSAPVKPGI.PYKQLTVGVPKEIFQNEKRVALSPAGVQALVKQGFVVVVESGAGEASKFSDDHYRAAGAQIQGAKEVLASDLVVKVRAPMLNPTL	GVH
E.coli E.tonalla	MRIGIPRERITNETEVAATEKTVEQLIKLGETVAVESGAGLASFDDKAPVQAGAEIV. EGNSVWQSEIILKVNAPL	
E. Cenerra R rubrum	REERVDPSSWF1FRRARVGVIDUS. NGSVNFVARFFVFRIRVAR VGIGFRVIVESGUSDASF1DELTRANGAVISCHUSTAVINGSVILLAV SAF	GTD
1.12.002.000	→a1	
	98	197
	+ + * + * * + * + * * + * * * * * * * *	***
B.taurus	EADLIKTSGTLISFIYFAQNPDILIKLSKRKTTVLAMQVPRVTIAQGYDALSSMANIAGYKAVVLAANHFGRFFTQGITAAGKVPPAKILIVGGV	AGL
E.COLL E tenella	ELALLAYGTILVSI INAQAPELMQAAAKAVI IMAMDSVEKI SKASSAMAALADI KAI VEAMDEGKEI IGUI HAAKAVPEAAKAVI IGASV Iyoo donkui. Isui donkaalaa haakava laanguval laanguval kangaakataviksamaa ahai kai veamaa kanga saada saakave k	AGL
R.rubrum	EVALIKEGAVIMCHLGALINRPVVEALTKRKITAYAMEIMPRISRAQSMDILSSQSNLAGYRAVIDGAYEFARAFPMMMTAAGTVPPARVLVFGVGV	AGL
	198	295
P. framers a	++ * +** * * * + *++ * * + + + + + + +	+**
E.coli	AATGAANSIGATVRAFTTRPEVKROVOSMAAEFLELD. FKREAGSGDGYAKVMSDAFTKAEMELFAAOAREVDIIVTALIPGKPAPKLITREMUD	SMK
E.tenella	QAISTAHGLGAQVFGHDVRSATREEVESCGGKFIGLRMGEEGEVLGGYAREMGDAYQRAQREMIANTIKHCDVVICTAAIHGRPSPKLISRDMLR	ISMK
R. rubrum	QAIATAKRIGAVVMATDVRAATKEQVESLGGKFITVDDEAMKTAETAGGYAKEMGEEFRKKQAEAVLKELVKTDIAITTALIPGKPAPVLITEEMVT	KMK
		387
B. taurus	KAN TAXA TAXA TAXA TAXA TAXA TAXA TAXA TA	T TVMK
E.coli	AGSVIVDLAAONGGNCEYTVPGEIFTTENGVKVIGYTDLPGRLPTOSSOLYGRNLVNLLKLLCKEKDGNITVDFDDVVIRGVT	VIR
E.tenella	$pgsvvvdlatefgdvrsgwggnvevspkddqivvd.gvtvigrrietrmpiqaselfsmnicnlledlgggsn\ldots frinddevirgivarder for the statemeter of th$	YAVY
R.rubrum	PGSVIIDLAVEAGGNCPLSEPGKI.VVKHGVKIVGHTNVPSRVAADASPLFAKNLLNFLTP.HVDKDTKTLVMKLEDETVSGTC	VTR
	222	400
	۰۰۰ مدر ۱۹۹۰ - ۲۰۰۰ ۲۰۰۰ ۲۰۰۰ ۲۰۰۰ ۲۰۰۰ ۲۰۰۰ ۲۰۰۰	403
B.taurus	DGQVIFPAPTPKNIPQGAPVKQKTVAELEAEKAATITPFRKTMTSASVYTAGLTGILGLGIAAPNLAFSQMVTTFGLAGIVGYHTWGVTFAL	HSP
E.coli	AGE ITWPAP. PIQVSAQPQAAQKAAPEVKTEEKCTCSPWRKYALMALAIILFGWMASVAPKEFLGHFTVFALACVVGYYVVWNVSHAL	HTP
E.tenella	QGRNVWQPSQPTPVSRTPPRGQMPPPSAPGAPAPEKPGAFAQALASDAFFAMCLVVAAAVVGLLGIVLDPVELKHLTLLGLSLIVGYYCVWAVTPSL	HTP
R. rubrum	DGAIVHPALTGQGA MEDCNILVEGFNQLSQQALELSQHAQALALQASHAV.LPAAAATEGASEFWWLMTVFVLACFIGFYVVWSVTPAL	HSP
	484	583
	********** **** * * * * * ****** *** *** *** ***	*
B.taurus	${\tt LMSVTNAISGLTAVGGLVLMGGHLYPSTTSQGLAALATFISSVNIAGGFLVTQRMLDMFKRPTDPPEYNYLYLLPAGTFVGGYLASLYSGYNIEQIM$	ſYLG
E.coli	LMSVTNAISGIIVVGALLQIGQGGWVSFLSFIAVLIASINIFGGFTVTQRMLKMFRKN MSGGLVTAA	VIV
E.tenella	LMSVTNALSGVIVIGGULEYGTAMISGFT.LLALIGTFLASVNVAGGFFVTRMLKMPQI MPSLIGAV	TYLF
R. Lubrum		1114
	584	683
	++ + +++ +++ +++ +++ +++ +++++++++++++	
B.taurus	SGLCCVGALASLSTQGTARLGNALGMIGVAGGLAATLGGLKPCPELLAQMSGAMALGGTIGLTIAKRIQISDLPQLVAAFHSLVGLAAVLTCIAEVI	IEY
E.COLL E feralla	AAILFIFSLAGLSKHETSROGNNFGIAGMAIALIATIFGPDTGNVGWILLAMVIGGAIGIRLAKKVEMTEMPELVALHSFVGLAAVLVGFNSIL GAICHTICTDGISTDORANGDUILGUVGUVANUMUTERAGGOUVII PEAMA.ADAICUVTAOSUMDEMDOIVAI PEAMA	
R. rubrum	Sate in the second set of the second second set of the second se	
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	684	1.1.NP
	684 +<	780
B.taurus E.coli	684 + + + + + + + + + + + + + + + + + + +	780 *
B.taurus E.coli E.tenella	684 + + + + + + + + + + + + + + + + + + +	780 * 7TLT VHLV
B.taurus E.coli E.tenella R.rubrum	664 + + + + + + + + + + + + + + + + + + +	780 * 7TLT WHLV FHLV
B.taurus E.coli E.tenella R.rubrum	664 + + + + + + + + + + + + + + + + + + +	780 * VTLT VHLV FHLV FLLI
B.taurus E.coli E.tenella R.rubrum	664 + + + + + + + + + + + + + + + + + + +	780 * VTLT VHLV FHLV FLLI 879
B.taurus E.coli E.tenella R.rubrum	664 + + + + + + + + + + + + + + + + + + +	780 * 71LT WHLV FHLV FHLV 879
B.taurus E.coli E.tenella R.rubrum B.taurus E.coli	684 + + + + + + + + + + + + + + + + + + +	TLNP 780 * TLT WHLV THLV TLLI 879 MIR SLLK
B.taurus E.coli E.tenella R.rubrum B.taurus E.coli E.tenella	684 + + + + + + + + + + + + + + + + + + +	780 * VTLT WHLV FHLV FLLI 879 MIR SLLK DELL
B.taurus E.coli E.tenella R.rubrum B.taurus E.coli E.tenella R.rubrum	684 + + + + + + + + + + + + + + + + + + +	780 * 71LT WHLV FHLV FHLV FHLV FHLV FHLV FHLV FHLV F
B.taurus E.coli E.tenella R.rubrum B.taurus E.coli E.tenella R.rubrum	684 + + + + + + + + + + + + + + + + + + +	780 * 780 * 71LT WHLV 71LI 879 MIR 879 MIR SLLK 20MIR
B.taurus E.coli E.tenella R.rubrum B.taurus E.coli E.tenella R.rubrum	664 + + + + + + + + + + + + + + + + + + +	780 * 71LT WHLV 71LI 879 MIR 879 MIR 21LK 21LK 21LK 21K 375
B.taurus E.coli E.tenella R.rubrum B.taurus E.coli E.tenella R.rubrum B.taurus	664 + + + + + + + + + + + + + + + + + + +	780 * 7117 7117 7117 7117 879 879 879 879 811K 979 211K 979 ** *
B.taurus E.coli E.tenella R.rubrum B.taurus E.coli E.tenella R.rubrum B.taurus E.coli	664 + + + + + + + + + + + + + + + + + + +	LNP 780 * 7TLT WHLV FHLV FHLV FHLV 879 0MIR 82LLK 879 0MIR 82LLK 975 975 * *
B.taurus E.coli E.tenella R.rubrum B.taurus E.coli E.tenella R.rubrum B.taurus E.coli E.tenella	684 + + + + + + + + + + + + + + + + + + +	LINP 7800 * TIT WHLV FILI 879 SMIR 821LK 975 975 975 * * * SPNS SPNS SPNS SPNS SPNS
B.taurus E.coli E.tenella R.rubrum B.taurus E.coli E.tenella R.rubrum E.taurus E.coli E.tenella R.rubrum	<pre>684 + + + + + + + + + + + + + + + + + + +</pre>	ALNP 780 * VTLT VHLV FHLV FHLV FHLV FHLV FHLV FHLV FHLV
B. taurus E. coli E. tenella R. rubrum B. taurus E. coli E. tenella R. rubrum B. taurus E. coli E. tenella R. rubrum	684 + + + + + + + + + + + + + + + + + + +	ALNP 780 * 71LT WHLV FHLV FHLV FHLV FHLV FHLV FHLV FHLV F
B.taurus E.coli E.tenella R.rubrum B.taurus E.coli E.tenella R.rubrum E.taurus E.coli E.tenella R.rubrum	664 + + + + + + + + + + + + + + + + + + +	TLINP 780 * TTLT WHLV FHLV FHLV 879 879 879 979 * * * * * * *
B.taurus E.coli E.tenella R.rubrum B.taurus E.coli E.tenella R.rubrum B.taurus E.coli E.tenella R.rubrum B.taurus	664 + <td>TLINP 780 * TTLT WHLV FHLV FHLV 879 879 DMIR 820 FLLI FILL FILL FILL FILL FILL FILL FIL</td>	TLINP 780 * TTLT WHLV FHLV FHLV 879 879 DMIR 820 FLLI FILL FILL FILL FILL FILL FILL FIL
B.taurus E.coli E.tenella R.rubrum B.taurus E.coli E.tenella R.rubrum B.taurus E.coli B.taurus E.coli	664 + <td>TTIT 780 * TTIT 780 * TTIT 879 * STLV 879 * STLV 879 * STLV 879 * STLV 879 * STLV * * * *</td>	TTIT 780 * TTIT 780 * TTIT 879 * STLV 879 * STLV 879 * STLV 879 * STLV 879 * STLV * * * *
B. taurus E. coli E. tenella R. rubrum B. taurus E. coli E. tenella R. rubrum B. taurus E. coli E. tenella R. rubrum B. taurus E. coli E. tenella R. rubrum	684 + <td>780 * 771LT * 771LT * 879 % % % % % % % % % % % % % % % % % % %</td>	780 * 771LT * 771LT * 879 % % % % % % % % % % % % % % % % % % %

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 αl , $\alpha 2$, 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-



Fig. 4. Immunoblotting of transhydrogenase subunits. The purified soluble subunit (α 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 (α 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 α 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.

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 Cterminal 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. 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 $\alpha 1$, $\alpha 2$, 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 $\beta\alpha\beta$ fold followed by an EEDQ-modifiable and NADH(and NMNH)protectable Glu₂₃₂, a FSBA-modifiable and NADHprotectable Tyr₂₄₅, and a DCCD-modifiable and NADH(and AMP)-protectable Glu₂₅₇ (Fig. 6). In the corresponding region of the α 1 subunit of the *R. rubrum* transhydrogenase, there is also a $\beta\alpha\beta$ fold (with the highest score of 11, see Wierenga *et al.*, 1986), and a conserved Tyr corresponding to Tyr₂₄₅ 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 α l 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 Glu₈₈₀ and a FSBA-modifiable Tyr₁₀₀₆. 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 Tyr₂₄₅ and Tyr₁₀₀₆ 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 $\alpha 1$ 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 ($\Delta E_{m,7} \simeq 5 \,\mathrm{mV}$) 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 Cys₈₉₃: 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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REFERENCES

- Capaldi, R. A., and Vanderkooi, G. (1972). Proc. Natl. Acad. Sci. USA 69, 930–932.
- Chen, E. Y., and Seeburg, P. H. (1985). DNA (N.Y.) 4, 165-170.
- Clarke, D. M., and Bragg, P. D. (1985). Eur. J. Biochem. 149, 517– 523.
- Clarke, D. M., Loo, T. W., Gillam, S., and Bragg, P. D. (1986). Eur. J. Biochem. 158, 647–653.
- Cunningham, I. J., Williams, R., Palmer, T., Thomas, C. M., and Jackson, J. B. (1992). Biochim. Biophys. Acta 1100, 332-338.
- Denhardt, D. T. (1966). Biochem. Biophys. Res. Commun. 23, 641– 646.
- Devereux, J., Haeberli, P., and Smithies, O. (1984). Nucleic Acids Res. 12, 387–395.
- Fisher, R. R., and Guillory, R. J. (1971). J. Biol. Chem. 246, 4687-4693.
- Hatefi, Y. and Yamaguchi, M. (1992). In Molecular Mechanisms in Bioenergetics (Ernster, L., ed.), Elsevier, Amsterdam, pp. 265– 281.
- Hou, C., Potier, M., and Bragg, P. D. (1990). Biochim. Biophys. Acta 1018, 61-66.
- Kramer, R. A., Tomchak, L. A., McAndrew, S. J., Becker, K., Hug, D., Pasamontes, L., and Hümbelin, M. (1993). *Mol. Biochem. Parasitol.* 60, 327–332.
- Kyte, J., and Doolittle, R. F. (1982). J. Mol. Biol. 157, 105-132.
- Laemmli, U. K., (1970). Nature (London) 227, 680-685.
- Lee, C. P., and Ernster, L. (1989). Biochim. Biophys. Acta 1000, 371-376.
- Lever, T. M., Palmer, T., Cunningham, I. J., Cotton, N. P. J., and Jackson, J. B. (1991). Eur. J. Biochem. 197, 247–255.
- McFadden, B. J., and Fisher, R. R. (1978). Arch. Biochem. Biophys. 190, 820-828.
- Mosbach, K., Guilford, H., Ohlsson, R., and Scott, M. (1972). Biochem. J. 127, 625–631.
- Olausson, T., Ahmad, S., Glavas, N., Bragg, P. D., and Rydström, J. (1992). EBEC Short Rep. 7, 31.
- Olausson, T., Nordling, M., Karlsson, G., Meuller, J., Lundberg, L., and Rydström, J. (1992). Acta Physiol. Scand. 146, 13-22.

- Ormerod, J. G., Ormerod, K. G., and Gest, H. (1961). Arch. Biochem. Biophys. 94, 449–463.
- Osawa, S., Muto, A., Ohama, T., Andachi, T., Tanaka, R., and Yamao, F. (1990). *Experientia* **46**, 1097–1106.
- Palmer, T., Williams, R., Cotton, N. P. J., Thomas, C. M., and Jackson, J. B. (1993). Eur. J. Biochem. 211, 663-669.
- Phelps, D. C., and Hatefi, Y. (1984a). Biochemistry 23, 4475-4480.
- Phelps, D. C., and Hatefi, Y. (1984b). Biochemistry 23, 6340-6344.
- Phelps, D. C., and Hatefi, Y. (1985). Biochemistry 24, 3503-3507.
- Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1988). Science 239, 487–491.
- Saito, H., and Miura, K.-I. (1963). Biochim. Biophys. Acta 72, 619-629.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: a Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Sanger, F., Nicklen, S., and Coulson, A. R. (1977). Proc. Natl. Acad. Sci. USA 74, 5463–5467.
- Shine, J., and Dalgarno, L. (1975). Nature (London) 254, 34-38.
- Southern, E. M. (1975). J. Mol. Biol.. 98, 503-517.
- Steinrücke, P., and Ludwig, B. (1993). FEMS Microbiol. Rev. 104, 83-118.
- Vermeulen, A. N., Kok, J. J., Van den Boogaard, P., Dijkema, R. and Claessens, J. A. J. (1993). FEMS Microbiol. Lett. 110, 223– 230.
- Wakabayashi, S., and Hatefi, Y. (1987a). Biochem. Int. 15, 667-675.
- Wakabayashi, S., and Hatefi, Y. (1987b). Biochem. Int. 15, 915-924.
- Wierenga, R. K., Terpstra, P., and Hol, W. G. (1986). J. Mol. Biol. 187, 101–107.
- Yamaguchi, M., and Hatefi, Y. (1989). Biochemistry 28, 6050-6056.
- Yamaguchi, M., and Hatefi, Y. (1991). J. Biol. Chem. 266, 5728– 5735.
- Yamaguchi, M., and Hatefi, Y. (1993). J. Biol. Chem. 268, 17871-17877.
- Yamaguchi, M., Hatefi, Y., Trach, K., and Hoch, J. A. (1988). J. Biol. Chem. 263, 2761–2767.
- Yamaguchi, M., Wakabayashi, S., and Hatefi, Y. (1990). Biochemistry 29, 4136-4143.