Cloning and sequencing of the genes for the proton-translocating nicotinamide nucleotide transhydrogenase from *Rhodospirillum rubrum* and the implications for the domain structure of the enzyme

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The genes for the proton-translocating nicotinamide nucleotide transhydrogenase from *Rhodospirillum rubrum* have been cloned using a probe constructed with the polymerase chain reaction, genomic DNA as target and oligonucleotide primers corresponding to amino acid sequence obtained from the purified soluble subunit. There is a cluster of three genes, designated *pntAA*, *pntAB* and *pntB*, whose translation products indicate polypeptides of 384, 139 and 464 amino acids, respectively. This contrasts with the situation in the enzymes from *Escherichia coli* (two polypeptides) and bovine mitochondria (one polypeptide) but there is close similarity between the sequences. PntAA is the soluble subunit of the enzyme from *R. rubrum*, equivalent to the relatively hydrophilic domain I that forms the N-terminal part of the α polypeptide of *E. coli* transhydrogenase and which probably contains the NAD(H)-binding site. PntAB corresponds to the strongly hydrophobic domain IIa at the C-terminus of the α polypeptide of the *E. coli* transhydrogenase. PntB corresponds to the *E. coli* β polypeptide, which comprises the strongly hydrophobic domain IIb and the relatively hydrophilic domain III, thought to contain the NADP(H)-binding site. The peptide bond between PntAA-Lys237 and -Glu238 of both the denatured and the native soluble subunit is very sensitive to proteolysis by trypsin and the neighbouring peptide bond Lys227-Thr228 to cleavage by the endoprotease Lys-C. Related sites have been reported to be sensitive to trypsin in the *E. coli* and bovine mitochondrial enzymes. The two tryptic fragments from the native *R. rubrum* soluble subunit are unable to reconstitute transhydrogenase activity to membranes depleted of the soluble subunit but they can block reconstitution by intact soluble subunit. It is suggested that this protease-sensitive region separates two subdomains and that, after trypsinolysis, at least one retains structural integrity and can dock with domains II and/or III.

Keywords: transhydrogenase, gene cloning, gene sequencing, proton translocation, *Rhodospirillum rubrum*

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Abbreviations: H⁺-Thase, proton-translocating, nicotinamide nucleotide transhydrogenase, EC 1.6.1.1; Th_s, the soluble subunit of transhydrogenase from *R. rubrum*; C₇-particles, chromatophores of *R. rubrum* depleted of the soluble transhydrogenase subunit by washing; IVR, inverted repeat sequence.

The GenBank accession number for the nucleotide sequence reported in this paper is U05294.

INTRODUCTION

Transhydrogenase (H⁺-Thase), from the inner membranes of animal mitochondria and the cytoplasmic membranes of many bacteria, couples the transfer of reducing equivalents between NAD(H) and NADP(H) to the translocation of protons:

Thus, in intact mitochondria and bacteria, the imposition of a transmembrane proton electrochemical gradient, for example through the action of a respiratory or photosynthetic electron-transport chain, drives the reaction to the right (Rydstrom *et al.*, 1987; Jackson, 1991; Hatefi & Yamaguchi, 1992).

The amino acid sequences of H⁺-Thase from *Escherichia coli* (Clark *et al.*, 1986; Ahmad *et al.*, 1992) and bovine mitochondria (Yamaguchi *et al.*, 1988) have been predicted from the nucleotide sequences of the genes (*pntA* and *pntB*) and the cDNA, respectively. The *E. coli* enzyme is composed of two polypeptides (molecular masses 54 kDa and 49 kDa). When their amino acid sequences are lined up contiguously (the C-terminus of α to the N-terminus of β), there is close similarity with the sequence of the single polypeptide (109 kDa) of the bovine enzyme (approximately 52% identity). Hydropathic profiles (Clarke *et al.*, 1986; Yamaguchi *et al.*, 1988) indicate that H⁺-Thase has three large domains. In the bovine enzyme the approximate positions of these domains are from residue 1 to 431 (domain I), from residue 432 to 836 (domain II) and from residue 837 to 1043 (domain III). In *E. coli* H⁺-Thase the 'break' between the two polypeptides is located in domain II. For reasons that will become clear, it is appropriate to consider that domain II is composed of two subdomains; the part of domain II on the α polypeptide of *E. coli* (or its homologues), will be denoted domain IIa, and the part of the β polypeptide (or its homologues) as domain IIb.

Domain II (both IIa and IIb) of H⁺-Thase is strongly hydrophobic and might comprise between 12 and 14 transmembrane helices (Clarke *et al.*, 1986; Yamaguchi *et al.*, 1988; Tong *et al.*, 1991). Domains I and III are relatively hydrophilic; they probably contain the NAD⁺/NADH-binding site and the NADP⁺/NADPH-binding site, respectively (Clarke *et al.*, 1986; Wakabayashi & Hatefi, 1987a; Yamaguchi *et al.*, 1988; Yamaguchi & Hatefi, 1993). The susceptibility of H⁺-Thase to proteases and the nature of the reaction with antibodies suggest that domains I and III both protrude on the matrix side of the membrane in bovine mitochondria (the cytoplasmic side in *E. coli*: Yamaguchi *et al.*, 1990; Yamaguchi & Hatefi, 1991; Tong *et al.*, 1991). It was shown (Cunningham *et al.*, 1992a) that there are strong sequence similarities (approximately 30% identity) between domain I of H⁺-Thase and the enzyme, alanine dehydrogenase (Kuroda *et al.*, 1990), which catalyses the reversible, reductive amination of pyruvate to alanine.

Recently, the translation of a sequence of cDNA isolated from human brain was found to be homologous with a part of domain IIb of bovine H⁺-Thase (Adams *et al.*, 1992; Kramer *et al.*, 1993). Sequence identity extends by two amino acid residues into the region which, in the bovine enzyme, forms the short polypeptide link between domains IIa and IIb. This indicates that the gene and polypeptide composition are similar in the two organisms. In contrast, the translation of a polymerase chain reaction product of genomic DNA from the protozoan parasite

Eimeria tenella indicates that this organism has an H⁺-Thase with a different polypeptide and gene structure (Kramer *et al.*, 1993). The full-length coding sequence establishes that H⁺-Thase from *Ei. tenella* comprises a single polypeptide, but that the domain order is different to that in the bovine enzyme; from the N-terminus of the protein it proceeds IIb > III > I > IIa. Thus, to observe sequence homology between *Ei. tenella* and *E. coli*, the two polypeptides of the bacterial enzyme must be aligned such that the C-terminus of the β polypeptides is contiguous with the N-terminus of the α . In that arrangement there is 45% amino acid identity between the sequences of H⁺-Thase from *E. coli* and *Ei. tenella* (Kramer *et al.*, 1993).

Another different polypeptide composition is suggested by analysis of H⁺-Thase from the photosynthetic bacterium *Rhodospirillum rubrum* (Cunningham *et al.*, 1992a). In this organism, the enzyme can be resolved, under mild conditions, into a water-soluble component (formerly known as Th_s) and a membrane-bound component (Fisher & Guillory, 1971a, b; Cunningham *et al.*, 1992a). Separately, neither component has transhydrogenase activity but the protein can be fully reconstituted in the presence of nucleotides (Cunningham *et al.*, 1992b). The N-terminal amino acid sequence and the molecular mass of the purified soluble transhydrogenase subunit indicated that it probably corresponds to domain I of H⁺-Thase (Cunningham *et al.*, 1992a). In this report we describe the sensitivity of Th_s to proteases, the cloning and sequencing of the genes for H⁺-Thase from *R. rubrum* and the implications for the domain structure of the enzyme.

METHODS

Bacterial strains and growth conditions. *R. rubrum* strain S1, from Dr L. Sloaten, Vrije Universiteit, Brussels, Belgium, was grown anaerobically under phototrophic conditions on RCV medium (Weaver *et al.*, 1975) at 30 °C in completely sealed bottles, as described by Cunningham *et al.* (1992a). *E. coli* strain MC1061 [*hsdR mcrB araD139 Δ(araABC-leu)7679 ΔlacX74 galK rpsL thi*; Meissner *et al.*, 1987] was grown with shaking in L-broth, or on L-agar (1.5%, w/v, agar added to L broth) at 37 °C, as described by Sambrook *et al.* (1989). Antibiotic resistance was selected by addition of 100 µg ampicillin ml⁻¹ to the solid medium.

Biochemical preparations and procedures. Chromatophores (everted membrane vesicles) of *R. rubrum* and C_T-particles (chromatophores washed to remove soluble transhydrogenase subunit) were prepared, and the soluble transhydrogenase subunit was purified and assayed, as described by Cunningham *et al.* (1992a). Crude soluble subunit (type II Th_s) was prepared as in Fisher & Guillory (1971a). Bacteriochlorophyll was assayed using the *in vivo* absorption coefficient (Clayton, 1963). Light-driven transhydrogenation was measured by following the reduction of thio-NADP⁺ with NADH at 395 nm using known absorption coefficients (Palmer & Jackson, 1992).

When N-terminal amino acid sequences of fragments were required, purified soluble transhydrogenase subunit was first subjected to SDS-PAGE. The 43 kDa polypeptide was excised, electro-eluted in 0.05% (w/v) SDS, diluted and incubated

overnight at 18 °C with protease (approximately 100:1 by mass). The samples were then concentrated, again subjected to SDS-PAGE and electro-blotted on to poly(vinylidene difluoride) membrane. Peptides were visualized with PAGE Blue-83, cut out and analysed with an Applied Biosystems 473A gas-phase sequencer. Further details of these procedures are described by Palmer *et al.* (1993). For the reconstitution experiments shown in Fig. 6, purified soluble subunit of *R. rubrum* H⁺-Thase (21 µg protein), dialysed against 1 mM dithiothreitol, 10 mM Tris/HCl pH 8.0, to remove salt and phenylmethylsulphonyl fluoride, was incubated for 5 h with 0.6 µg trypsin in a volume of 0.9 ml. The reaction was stopped with 6 µg trypsin inhibitor.

In vitro manipulation and analysis of DNA. Genomic DNA from *R. rubrum* was prepared by a modification of the method of Fitzmaurice *et al.* (1989), with the following exceptions. The 80 ml overnight culture was grown phototrophically (see above). The bacterial cells, harvested by centrifugation, were washed in 20 mM EDTA, 50 mM Tris/HCl pH 8.0, resuspended in 10 ml of the same medium supplemented with 0.5% SDS and 2.5 mg RNase, and incubated at 37 °C for 1 h. Proteinase K (2.5 mg) was added and the incubation was continued at 65 °C for 3 h. The lysate was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) and then twice with chloroform/isoamyl alcohol (24:1, v/v). Following addition of sodium acetate, the DNA was precipitated with ethanol, removed by spooling, resuspended in sodium acetate, reprecipitated and washed in ethanol.

Plasmid DNA was prepared on both small and large scales by the alkaline-SDS method (Birnboim & Doly, 1979) and purified, where necessary, by caesium chloride/ethidium bromide equilibrium density-gradient centrifugation. DNA manipulations and the handling of bacteria were carried out by standard techniques (Sambrook *et al.*, 1989). Restriction enzymes were used according to the manufacturers' instructions. Oligonucleotide probes were labelled with [α -³²P]dCTP, as described

by Feinberg & Vogelstein (1984). DNA sequencing was performed both by the dideoxy-chain-termination method (Sanger *et al.*, 1977) with a Sequenase version 2.0 kit from United States Biochemicals, and with an Applied Biosystems Incorporated 373A automated sequencer using the manufacturer's kit employing *Taq* polymerase and dye terminators according to their instructions.

Computer alignments, using the University of Wisconsin GCG package (Devereux *et al.*, 1984), and searches, were carried out on the Owl database at the Science and Engineering Research Council Facility at Daresbury, and through the Biocomputing Unit at the University of Edinburgh. Hydropathic profiles were determined using a program written by Dr D. A. Rouch, School of Biological Sciences, University of Birmingham, based on the parameters described by Von Heijne (1992).

Cloning of genes for H⁺-Thase from *R. rubrum*. On the basis of N-terminal sequence of Th_s and peptides prepared by proteolysis of Th_s (Cunningham *et al.*, 1992a), degenerate oligonucleotides were synthesized as primers for the polymerase chain reaction (PCR) using genomic DNA from *R. rubrum* as a target. The products were re-amplified and sequenced. Comparison with published sequences confirmed that the PCR products were derived from H⁺-Thase. A restriction map of *R. rubrum* DNA, using labelled PCR product as a probe of Southern blots, is shown in Fig. 1. *Bam*HI fragments of *R. rubrum* DNA between 4 and 7 kb were cloned into pBR322 and used to transform *E. coli* MC1061. Clones bearing inserts were selected, by colony hybridization, again using labelled PCR product as a probe. Four positive clones were selected, and shown by restriction analysis to contain DNA equivalent to the PCR product. One of these, pNIC1, was used for nucleotide sequencing (see Fig. 1). On the basis of the restriction map of this construct, DNA fragments were subcloned into pUC18 and nucleotide sequences of the 5' and 3' ends of the fragments were determined using universal forward and reverse primers. From these sequences oligonucleotides were constructed to serve as

Fig. 1. (a) Restriction map of the 6.1 kb *Bam*HI insert of *R. rubrum* DNA obtained by colony hybridization, and (b) the sequencing strategy. E, *Eco*RI; B, *Bam*HI; S, *Sal*I; H, *Hind*III. The solid arrows to the left and right show sequences obtained for the top and bottom DNA strands, respectively. The direction of the arrows show the direction of sequencing and the position of the arrows indicates regions over which sequence was obtained using universal forward and reverse, or newly-synthesized primers (see Methods). The dashed arrows indicate the position of the three H⁺-Thase genes. The numbers of base pairs between the structural genes and the *Bam*HI sites are only approximate.

primers for further sequencing using either pNIC1 or appropriate subclones in pUC18.

Materials. Nicotinamide nucleotides, TPCK-treated trypsin (T8642) and soybean trypsin inhibitor (T9003) were obtained from Sigma, sequencing-grade Lys-C and Asp-N endoproteinases from Boehringer, restriction enzymes and ligases from Northumbria Biologicals, radiolabelled nucleotides from Amersham, and oligonucleotides from Alta Bioscience.

RESULTS AND DISCUSSION

Nucleotide sequence of *R. rubrum* *pnt* genes reveals novel gene organization

The nucleotide sequence from the cloned *Bam*HI fragment, and its translation product, are shown in Fig. 2. The *pnt* genes were assigned by locating regions coding for polypeptides containing sequence identical to that of proteolytic fragments of the soluble subunit of *R. rubrum* H⁺-Thase, and by similarity to the *E. coli* *pnt* genes. There are three ORFs, which we have designated *pntAA*, *pntAB* and *pntB* (reasons for this nomenclature will become evident below). Each is preceded by reasonable translational signals and is separated from the adjacent ORF by only 3 and 13 nucleotides, respectively. Downstream of *pntB* are two IVRs, both of which are potential *rho*-independent transcriptional terminators, since they consist of a G + C-rich hairpin, followed by a run of A + T-rich sequence (Platt, 1986). Upstream of *pntAA* is a region of at least 103 bp which is unlikely to be translated because of the presence of stop codons in all three frames. This region also contains two IVRs which may be involved with transcriptional termination (Platt, 1986) but could also be binding sites for regulatory proteins. These upstream and downstream features probably thus define the *pnt* genes as a translational unit containing three cistrons. Upstream of *pntAA* there is no obvious sequence to fit a consensus for either E σ^{70} or E σ^{54} (Harley & Reynolds, 1987; Kustu *et al.*, 1989) which can identify the likely promoter, although there clearly would be space for an RNA polymerase binding site. The expression and regulation of these genes will form the basis of a separate study.

The predicted molecular masses of the gene products PntAA, PntAB and PntB are 40.3, 14.9 and 47.8 kDa, respectively. Analysis and comparison of the predicted amino acid sequences clearly reveal that, in contrast to the one and two polypeptides of bovine mitochondrial and *E. coli* transhydrogenases, respectively, the enzyme from *R. rubrum* consists of three polypeptides (Fig. 3). Thus, the α polypeptide of H⁺-Thase of *E. coli* can be viewed as a fusion of the PntAA and PntAB polypeptides of the *R. rubrum* enzyme (from the C-terminus of PntAA to the N-terminus of PntAB); the β polypeptide of *E. coli* and the PntB polypeptide of *R. rubrum* are equivalent. On this basis, and on the basis of the previously established relationship between the *E. coli* and bovine enzymes, the alignment shown in Fig. 4 indicates that there is 49.6% amino acid identity between the *E. coli* and *R. rubrum* H⁺-

Thases and 43.4% between the *R. rubrum* and bovine mitochondrial proteins (not shown). There is greater sequence divergence between the *R. rubrum* enzyme and the *E. coli* and the bovine enzymes than between the *E. coli* and bovine enzymes (52% identity). This divergence arises mainly in the region of the PntAA polypeptide of *R. rubrum*.

Subunit properties predicted from the Pnt primary amino acid sequences

Hydropathic profiles of the predicted polypeptides of *R. rubrum* H⁺-Thase are shown in Fig. 5. Except for the region of the NAD(H)-binding site (see below), PntAA generally has a rather hydrophilic character. Comparison of the N-terminal and internal sequences with the amino acid sequence predicted from the gene establishes unequivocally that this gene product is Th_s. The possibility that Th_s is a proteolytic breakdown product of H⁺-Thase, which formerly could not be eliminated (Cunningham *et al.*, 1992a) can now be discounted. Thus, uniquely in *R. rubrum*, domain I can be displaced as a discrete, water-soluble protein by washing from the chromatophore membrane. Interestingly, the region between domains I and II in H⁺-Thase of *E. coli* and bovine mitochondria is sensitive to proteolysis by trypsin (Tong *et al.*, 1991; Yamaguchi *et al.*, 1990) and it is possible that, in those systems under non-denaturing conditions, the resulting 43 kDa polypeptides might reconstitute transhydrogenase activity to depleted membranes.

PntAB is a small hydrophobic polypeptide. In the equivalent region (domain IIa) of the bovine and *E. coli* enzymes, four transmembrane helices were predicted (Yamaguchi *et al.*, 1988; Yamaguchi & Hatefi, 1991; Tong *et al.*, 1991). Four helices are also indicated in a hydropathy analysis of the sequence from Kramer *et al.* (1993) of *Ei. tenella* transhydrogenase (data not shown). However, the hydropathy profile shown in Fig. 5, which uses the scale of Von Heijne (1992), predicts, with reasonable confidence, that only the last three of these segments are transmembranous in *R. rubrum* PntAB. In an analysis using the amalgamated hydropathy scale of Kyte & Doolittle (1982), the first putative transmembrane helix (from Ala25 to Thr43) is again only weakly predicted in PntAB (data not shown). If it is assumed that the arrangement of transmembrane helices in the four transhydrogenases is similar, then, in view of the well-conserved cluster of positive charges at the C-terminus of PntAB (residues 129–139), which, by the 'positive-inside rule' should be located on the cytoplasmic side of the membrane (Von Heijne, 1992), and the fact that domain I is assuredly cytoplasmic (Cunningham *et al.*, 1992a), an even number of transmembrane helices in the polypeptide is most likely. Provisionally therefore, it is suggested that the first, weakly predicted transmembrane helix of PntAB should be accepted. It cannot be excluded that the transmembrane helix composition of PntAB is different to the equivalent region in other species but, if there were only three transmembrane helices, the N-terminus of PntAB would probably be located in the periplasm, which

<p>1 TTTCCGGTCGACCGTCTTGTGTGCGGATGGGGGAACGGGCATGGTTCCGCCCTTGGGCT</p>	<p>SD 1681 AGGGGCACGCGACCATGACGCATAGTCTTACCATTGGCGGCCTATATCGTCGCCCGGCTGC M T H S L T M A A Y I V A G V L</p>
<p>61 CGATAAGACCTTAGACCGTCTCACGACAATTGATGGGTGCGTTATGAAGATCGCTATTCC</p> <p>SD pntAA M K I A I P</p>	<p>1741 TCTTCATTCTCGCCCTGCGCGGCTGTCCAATCCGGAATCGGCGCGCAACGGCAACCGCA F I L A L R G L S N P E S A R N G N R M</p>
<p>121 AAAAGAGCGACGCCCCGGCGAGGATCGCGTCGCCATCTCTCCCGAGGTGGTGAAGAAGCT</p> <p>K E R R P G E D R V A I S P E V V K R L</p>	<p>1801 TGGGCATGGTCGGCATGGCGATCGCCATCCTGACCACGCTGCTGTGCGCTTCGGTTCAGG G M V G M A I A I L T T L L S P S V Q A</p>
<p>181 CGTCGGCTGGGGTTCGAGGTGATCGTGAACAAGGGCGCGGTGTCGGCGCGTCGATAAC</p> <p>V G L G F E V I V E Q G A G V G A S I T</p>	<p>1861 CCTATGCTGGATCGTCTGGCGATCGCCATCGCGCGGCCCATCGCACGCGTATCGCCA Y A W I V L A I A I G G A I G T V I A K</p>
<p>241 CGACGATGCTTTGACCGCGCGCGGCCACCATCGCCAGCAGCGCGCGCAGGCCCTGTC</p> <p>D D A L T A A G A T I A S T A A Q A L S</p>	<p>1921 AAAAGTGTGATGACCGCCCTGCCGAGCTTGTGCGCGCTTCCACTCGTGTGTCGGCA K V L M T A L P Q L V A A F H S L V G M</p>
<p>301 CCAGGCGGATGTGGTCTGGAAGGTACAGCGCGCGATGACCCGAGGAGGSCACCGACGA</p> <p>Q A D V V W K V Q R P M T A E E G T D E</p>	<p>1981 TGGCGCGGTGCTGGTGGCCACCGCGCCCTGCTCAATCCGAAGCCTATGGCATCGGT A A V L V A T G A L L N P E A Y G I G S</p>
<p>361 GGTGCGCTGATCAAGGAAGCGCGGTCTGATGTGCCATCTTGGCGCCTGACCAACCG</p> <p>V A L I K E G A V A L M C H L G A L T N R</p>	<p>2041 CGGCGGGGCGATCCACGCCGGATCGCTGCTGAAATGTCGCTCGGCTGGCGCTCGGCG V A L I H A G S L V E M A G I G L S P S V Q A</p>
<p>421 TCCGGTGGTTCGAGGCGCTGACCAAGCGCAAGATCACCAGCTATGCCATGGAATGATGCC</p> <p>P V V E A L T K R K I T A Y A M E L M P</p>	<p>2101 CCATCACCTTCTCGGGATCGGTGATCGCCTTGGCAAGCTTCAGGGGCTGATCGCGGCA I T F S G S V I A F G K L Q G L I A G K</p>
<p>481 GCGGATCAGCGCGCGCCAGTTCGATGATATCTTTCAGCGAGTCGAACCTCGCGGGCTA</p> <p>R I S R A Q S M D I L S S Q S N L A G Y</p>	<p>2161 AGCGGTGACCTTCCGATGCGAGCATCCGCTGAACGCCGTGCTTGGCATTCTGCTGGTGC P V T F P M Q H P L N A V L G I L L V V</p>
<p>541 TCGCGCGTGTGATGACGCGCGCTTATGAATTGCGCGCGCGCTTCCGATGATGATACCGC</p> <p>R A V I D G A Y E F A R A F P M M M T A</p>	<p>2221 TGCTGCTGGTGGTCTTCGCGCGCCACCGAAAGCCACCGCCTATTTCGCGCTGATGATCC L L V V F A A T E S H T A Y F A L M I L</p>
<p>601 CGCGCGCACCGTGC CGCGCGCGCGCGTGTGTTGGCGTTCGCGCGTTCGCGGATGCA</p> <p>A G T V P P A R V L V F G V G V A G L Q</p>	<p>2281 TGGCCTTCGCGCTTGGCTTCTGCTGATCATTCCGATCGCGCGCGCGACATGCCGCTG A F A L G F L L I I P I G G A D M P V V</p>
<p>661 GCGATCGCCACCGCAAGCGCTGGGCGCGGTGTCGTCACCGACGCTTTCGCGCGCG</p> <p>A I A T A K R L G A V A T V M A T D V K R A</p>	<p>2341 TCATCTGATGCTCAACAGCTATTCTGGGCTGGGCGGCTGCGGGCATTGGCTTACCGTGG I S M L N S Y S G W I A A G I G D F T L G</p>
<p>721 CACCAAGGAACAGGTGGAAGCTTGGGCGGCAAGTTCATCACCGTTCGATGACGAGCGAT</p> <p>T K E Q V E S L G G K F I T V D D E A M</p>	<p>2401 GCAATCCGCTGCTGATCATCGCGCGCGCTGCTGGGCTGCTGCGGCGCATCTCTCAGCT N P L L I I A G A L V G S S G A I L S Y</p>
<p>781 GAAAGCCGCGAGACCGCGCGCGCTACGCCAAGGAAATGGCGAGGAGTCCGCAAGAA</p> <p>K T A E T A G G Y A K E M G E E F R K K</p>	<p>2461 ACATCATGTGCAAGGCGATGAACCGCTCGATCTTCAACGTCATCCTGGGCGGCTTCGGCA I M C K G M N R S I F N V I L G G F G S</p>
<p>841 GCAGGCGGAGCGCGTCTCAAGAACTGGTCAAGACGATATCGCCATCACCACCGCCCT</p> <p>Q A E A V L K E L V K T D I A I T T A L</p>	<p>2521 GCGAGGGCGCGTAGCGCGCGCGGTGGCGCGCGCGCGCATGCTTGGTCAAGCGCGCA E G G V A A A G G A A G D R S V K A G S</p>
<p>901 GATCCCGCGCAAGCGCGCGCGGTGCTGATCACCAGGAGATGGTGAACAGATGAAGCC</p> <p>I P G K P A P V L I T E E M V T K M K P</p>	<p>2581 GCGCGGAAGACGCGCGCTTCATCATGAAGAACCGCTCGAAGGTTCATCATCGTGC CGCGT A E D A A F I M K N A S K V I I V P G Y</p>
<p>961 GGGCAGCGTCATCATGATGCTGGCGCTGGAAGCGCGCGCAATGGCCGCTGCTCGGAGCC</p> <p>G S V I I D L A V A E A G G N C P L S E P</p>	<p>2641 ATGGCATGGCGGTGGCG G M A V A Q A Q A H A L R E M A D G F T L K K</p>
<p>1021 GGGCAAGATCGTTGTCAACACGCGCTCAAGATCGTTGGCCACCAACGTCGCCCTCGCG</p> <p>G K I V V K H G V K I V G H T N V P S R</p>	<p>2701 AGGAAGGCGTCGAGGTTTCTTACGCCATCCATCCGCTGGCGCGCGCGCGCGCGCGCGCG E G V E V S Y A I H P V A G R M P G H M</p>
<p>1081 CGTCGCGCGCGACGCCACCGCTTGTTCGCCAAGAACCTTCTGAACCTTCTCAACCCCGCA</p> <p>V A A D A S P L F A K N L L N F L T P H</p>	<p>2761 TGAACGTGCTGCTGGCGGAGGCAATGTGCCCTATGACGAGGTCTTCGAGCTCGAAGAGA N V L L A E A N V P Y D E V F E L E E I</p>
<p>1141 CGTCGCAAGGACACGAGCGCTGATGAAGCTCGAGGACGAAACGCTTTCGGGCGAC</p> <p>V D K D T K T L V M K L E D E T V S G T</p>	<p>2821 TCAACAGCTCGTTCAGACCGCGCGATGTCGCTTCTGTCATCGCGCGCAACGACGTGACCA N S S F Q T A D V A F V I G A N D V T N</p>
<p>1201 CTGTGTGACCGCGATGGCGCGCATCGTCCATCCGCGCGTACCGGACAGGGAGCATAAGC</p> <p>C V T R D G A I V H P A L T G Q G A *</p>	<p>2881 ACCCGCGCGCAAGACCGATCCGTCGAGCGCGATCTACGGCATCGCCGCTTTCGAGCTTG P A A K T D P S S W I A A G M I G F L D V E</p>
<p>1261 GATGGAAGACAAGAACATCTCTGTCGAGGCTTCAATCAGCTCTCGCAACAGGCGCTTGA</p> <p>M E D K N I L V E G F N Q L S Q L A L E</p>	<p>2941 AAAAGCGCGAACCGCTGCTTCAACGCTCGATGGCGCTCGGGCTATGCGCGCTCG K A G T V L F I K R S M A S G Y A G V E</p>
<p>1321 ATTGTCCAGCATGCCAGCGCTTGGCGCTTCAAGCGCCATCGGTTCTGCGCGCGC</p> <p>L S Q H A Q A L A L Q A S H A V L P A A</p>	<p>3001 AGAACGAACTGTTCTTCCGCAACACAGCATGATGCTGTTGGCGAGCGCAAGAGATGA N E L F F R N N T M M L F G D A K K M T</p>
<p>1381 CGCGCGCACGGAAGCGCGCGAGTCTTGGTGGCTGATGACGCTTTCGCTCGTGGCTG</p> <p>A A T E G A S E F W W L M T V F V L A C</p>	<p>3061 CCGAGCAGATCGTCCAGGCGATGAACGCTGACCGCTTGAGAGCGGTATTGATTACGAAAA E Q I V Q A M N *</p>
<p>1441 CTTTCATCGCTTTTATGTGTGTGGTGGTACCGCGCGCTTTCAGCGCGCTGATGGG</p> <p>F I G F Y V V W S V T P A L H S P L M G</p>	<p>3121 GGGCGGGACCGCGACGCGTCCCGCGCTTTCGCGGATTAGG</p>
<p>1501 CGTCACCAACGCCATTTCCTCGTTCATCGTCTCGGGCGCTGATCGCCACCGCTCTGA</p> <p>V T N A I S S V I V V G A L I A T G P E</p>	
<p>1561 AGCCTTCAGCGCTGGAAGGTGCTGGGCTTCTTCGCCATCTGCTTTCGAGCGTGAACAT</p> <p>A F S A S K V L G F F A I L L A S V N I</p>	
<p>1621 CTTGCGCGGTTTCATCGTACCCCAACGAATGCTGGCATGTTCAAGAAGAAGCAGAAGTA</p> <p>F G G F I V T Q R M L A M F K K K Q K *</p>	

Fig. 2. Nucleotide sequence of the *pntAA*, *pntAB* and *pntB* genes of H^+ -Thase from *R. rubrum* and their predicted translation products. Probable Shine-Dalgarno sequences are indicated by horizontal lines above the nucleotide sequence. Inverted repeats are indicated by pairs of arrows. The N-terminal sequences of the soluble subunit of H^+ -Thase and its proteolytic fragments (Cunningham *et al.*, 1992a) are shown with solid lines under the amino acid sequence. The sequence of one of the proteolytic fragments, DILSSQSNLAGYRAV, obtained with endoproteinase Asp-N, has not been previously published.

Fig. 3. Comparison of the domain and polypeptide structures of H^+ -Thase from *R. rubrum*, *E. coli*, bovine mitochondria and *Ei. tenella* and with alanine dehydrogenase from *Bacillus stearothermophilus*. Unshaded domains are relatively hydrophilic; shaded domains are strongly hydrophobic. The dashed line linking domains III and I of the *Ei. tenella* protein represents a linker of 38 amino acid residues (Kramer *et al.*, 1993).

seems unlikely as it does not possess a recognizable signal sequence (see Von Heijne, 1986).

PntB, homologous to the β subunit of *E. coli* H^+ -Thase, has a strongly hydrophobic domain IIb (Fig. 5), and a relatively hydrophilic domain III, which, by analogy, probably protrudes from the membrane on the cytoplasmic side (Yamaguchi *et al.*, 1990; Yamaguchi & Hatefi, 1991; Tong *et al.*, 1991). Using the analysis of Kyte & Doolittle (1982), eight transmembrane helices were predicted for the *E. coli* β subunit (Tong *et al.*, 1991). The equivalent helices are clearly recognizable in the hydropathic profile of PntB of *R. rubrum* (Fig. 5). If the conclusion (above) is correct, that PntAB has four transmembrane helices, and if the assumption, that the bovine, *E. coli* and *R. rubrum* enzymes have a similar arrangement of transmembrane helices, is to be maintained, then an even number of helices for PntB (and *E. coli* β subunit) is indeed to be expected. It was predicted that the homologous region of bovine mitochondrial H^+ -Thase has ten transmembrane helices (Yamaguchi *et al.*, 1988; Yamaguchi & Hatefi, 1991). One of these was proposed within the sequence, unique to bovine H^+ -Thase, that forms the link between the equivalent of *R. rubrum* PntAB and PntB (or the α and β subunits of the *E. coli* enzyme). The sequence is also absent in H^+ -Thase from *Ei. tenella* (Kramer *et al.*, 1993) and the suggestion that it constitutes a transmembrane helix should perhaps be treated with some reservation. The other 'extra' putative transmembrane helix in mitochondrial H^+ -Thase (Yamaguchi *et al.*, 1988; Yamaguchi & Hatefi, 1991) can, in fact, be accommodated in the hydropathic profiles of the *E. coli* β and the *R. rubrum* PntB polypeptides (e.g. residues 215–233 in the latter). However, an odd number of transmembrane helices would challenge our assumption

that the arrangement is similar in all the known enzymes. The dilemma is heightened by a consideration of the charged residues in predicted loops lying between putative transmembrane helices. Thus, only one (148–164 in PntB) has a preponderance of Arg and Lys residues in H^+ -Thase from all four species. Taking PntB from *R. rubrum* in isolation, only two of the possible loops in domain IIa have net positive charge (one, adjacent to the two Lys residues at positions 76 and 77, and the other at residues 148–164). Assuming that these loops, the N-terminus and the C-terminus of PntB are all cytoplasmic, this fits comfortably with the inclusion (at residues 215–233) of the 'extra' transmembrane helix described by Hatefi and colleagues in the mitochondrial enzyme (see above). However, it would also require either that one of the first four predicted transmembrane helices, identified in the hydropathy profile, is in error, or that the N-terminus of the polypeptide is periplasmic rather than cytoplasmic (though it has no recognizable signal sequence). Clearly more evidence is required before firm conclusions can be drawn.

The membrane-bound component of H^+ -Thase from *R. rubrum* proved difficult to purify (Palmer *et al.*, 1993), partly because it is a relatively minor protein in chromatophore membranes, but also because of the very stringent detergent requirement to achieve solubilization without inactivation (the ability to reconstitute transhydrogenase activity with the water-soluble subunit): possibly PntAB is easily separated from PntB by many of the commonly used detergents. In routine preparations of the membrane-bound component of *R. rubrum* H^+ -Thase, polypeptides with apparent molecular mass of 50 kDa, 52 kDa and 56 kDa were observed on SDS-PAGE (Palmer *et al.*, 1993), and are likely candidates for PntB, but they were

obtained in insufficient quantities for N-terminal sequencing. Polypeptides with a molecular mass corresponding to that of PntAB were not detected on SDS-PAGE, again indicating that this component might be easily lost during purification.

There is presently considerable interest in the nature and location of the NAD(H)- and the NADP(H)-binding sites on H⁺-Thase. The β - α - β Rossmann fold, characteristic of ADP/FAD binding sites (Wierenga *et al.*, 1986), and thought to represent the NAD(H)-binding site in H⁺-Thase (Clarke *et al.*, 1986; Yamaguchi *et al.*, 1988), is strongly conserved in PntAA, centred on the Gly-X-Gly-X-X-Gly motif at residues 179–184. Tyr245 in mitochondrial H⁺-Thase reacts with the NAD(H) analogue [*p*-(fluorosulphonyl)benzoyl]-5'-adenosine (Wakabayashi & Hatefi, 1987a) and is conserved in PntAA (Tyr235),

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Fig. 5. Hydropathic profiles of the PntAA, PntAB and PntB polypeptides of H^+ -Thase from *R. rubrum* derived using the parameters described by Von Heijne (1992) – see Methods. A value of 0.05 is added to each hydrophobicity score to scale the plot so that shaded regions indicate putative transmembrane helices.

domain I, just downstream of the NAD(H)-binding site and rich in polar and charged residues (Fig. 5), corresponds to a flexible loop which is susceptible to modification by proteases and, in some conditions, by dicyclohexylcarbodiimide and [*p*-(fluorosulphonyl)benzoyl]-5'-adenosine. Thus, the peptide bond C-terminal to a conserved Lys (residue 237 in PntAA) is sensitive to trypsin in the enzyme from *R. rubrum* (Cunningham *et al.*, 1992a), *E. coli* (Tong *et al.*, 1991) and bovine mitochondria (Yamaguchi *et al.*, 1990). Furthermore, in PntAA a neighbouring peptide bond, Lys227-Thr228, is very sensitive to the endoprotease Lys-C (apparently not investigated in the *E. coli* and bovine enzymes). When the native purified soluble subunit of *R. rubrum* H^+ -Thase was treated with concentrations of trypsin low enough to give only two polypeptides on SDS-PAGE (apparent molecular mass 29 kDa and 19 kDa, thus corresponding to cleavage at Lys237-Glu238), with only minimal contamination from other fragments, its capacity to reconstitute transhydrogenase activity to depleted chromatophore membranes was severely inhibited. However, the data of Fig. 6 show that the trypsin-treated protein blocked reconstitution by untreated material. Thus, a crude preparation of soluble subunit was used to titrate light-driven transhydrogenation to about 90% of full activity (the 'crude' enzyme was used because pure protein is difficult to prepare in substantial quantities; see Cunningham *et al.*, 1992a). As expected, further addition of pure soluble subunit led to complete reconstitution. However, in contrast, further addition of the trypsinized material led to inhibition of transhydrogenation. Evi-

Fig. 6. The two tryptic fragments of the soluble subunit of H^+ -Thase from *R. rubrum* block reconstitution of light-driven transhydrogenation by the intact subunit and depleted chromatophore membranes. In (a), C_T -particles (bacteriochlorophyll to a final concentration of 10 μM) in 2.5 ml 125 mM sucrose, 2.7 mM MgCl_2 , 60 μM thio-NADP $^+$, 250 μM NADH, 44 mM Tris/HCl pH 8.0, were incubated for 1–2 min with a crude preparation ('type II') of soluble subunit (10 mg protein ml^{-1}), as shown, and then assayed for light-driven transhydrogenation at 30 °C. The incubation conditions indicated by the arrow were used as a basis for the experiments shown in (b). Here, additional purified soluble subunit (30 μg protein ml^{-1}), either trypsin-treated (see Methods) or untreated, was added, as shown. In control experiments, (i) addition of trypsin and trypsin-inhibitor to C_T -particles in the presence of crude soluble protein to give 90% reconstitution, but in the absence of purified protein, decreased the rate of transhydrogenation by only 7%, (ii) 50 μl of untreated, purified soluble subunit added to C_T -particles (in the absence of crude protein) gave transhydrogenation rates of 0.90 μmol (bacteriochlorophyll) $^{-1}$ min^{-1} , and (iii) 50 μl of trypsin-treated, purified soluble subunit added to C_T -particles (in the absence of pure protein) gave transhydrogenation rates of 0.37 μmol (bacteriochlorophyll) $^{-1}$ min^{-1} .

dently, at least one of the two polypeptide fragments retains structural integrity and is capable of docking with the membrane component of the enzyme, thus competing with the intact protein for binding sites. It proved difficult to separate the 19 kDa and 29 kDa polypeptides, even by HPLC, with the small amounts of material that are available.

Information on the location of the NADP(H)-binding site of H^+ -Thase is rather less clear, although it seems to be in domain III of the enzyme (Yamaguchi & Hatefi, 1993). The Gly residue at β 314, which is replaced by a Glu in the RH1 mutant of *E. coli* H^+ -Thase (Ahmad *et al.*, 1993), is conserved in the *R. rubrum* enzyme (PntB-Gly315). In the RH1 mutant, the influence of NADP(H) on the pattern of polypeptides generated by trypsin treatment is lost (Ahmad *et al.*, 1993). The Tyr residue, which has been implicated in NADP(H) binding because of its reactivity in the mitochondrial enzyme with [*p*-(fluorosulphonyl)benzoyl]-5'-adenosine (Wakabayashi & Hatefi, 1987a) and 8-azido-AMP (Hu *et al.*, 1992), despite the fact that, strictly, both are NAD(H) analogues, is conserved in

R. rubrum H⁺-Thase (PntB-432). Note that replacement of this residue by site-directed mutagenesis shows that it is not essential (Olausson *et al.*, 1993).

Evolutionary relationships between alanine dehydrogenase and H⁺-Thase

A strong sequence similarity between the alanine dehydrogenases of two species of *Bacillus* and domain I of the H⁺-Thases of *E. coli* and bovine mitochondria was previously reported (Cunningham *et al.*, 1992a). There is 29% identity between PntAA from *R. rubrum* and alanine dehydrogenase from *Bacillus stearothermophilus* (Kuroda *et al.*, 1990 – see Fig. 3).

It is evident that, despite the different polypeptide composition, the order of translation of the domains within the transcriptional unit of H⁺-Thase is the same in the *R. rubrum*, *E. coli* and bovine systems (I > IIa > IIb > III) but is different in *Ei. tenella* (IIb > III > I > IIa; Kramer *et al.*, 1993). It is likely that gene fusions and rearrangements have given rise to the different organizations. In view of the sequence similarity between domain I and alanine dehydrogenase, it is possible that the *R. rubrum* structure represents the most primitive of those studied so far. Thus, following suggestions for the evolution of other membrane proteins (Walker, 1992), we suppose that an *R. rubrum*-like H⁺-Thase would have originated from an association between an ancestor of alanine dehydrogenase (becoming domain I) with an ancestor of the membrane-located domain IIa and domain IIb/III proteins. Subsequent clustering of the genes and fusion between domains I and the IIa protein would have given the *E. coli* organization, and further fusion between domains IIa and IIb would have given the bovine organization. A different gene clustering arrangement or a transposition of the gene segments corresponding to domain I-IIa and domain IIb-III followed by fusion would have given an *Ei. tenella*-like protein.

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