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The *pet* genes of *Rhodospirillum rubrum*: cloning and sequencing of the genes for the cytochrome bc_1-complex

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Summary. A cytochrome bc_1-complex of *Rs. rubrum* was isolated and the three subunits were purified to homogeneity. The N-terminal amino acid sequence of the purified subunits was determined by automatic Edman degradation. The *pet* genes of *Rhodospirillum rubrum* coding for the three subunits of the cytochrome bc_1-complex were isolated from a genomic library of *Rs. rubrum* using oligonucleotides specific for conserved regions of the subunits from other organisms and a heterologous probe derived from the genes for the complex of *Rb. capsulatus*. The complete nucleotide sequence of a 5500 bp *Sal*I/*Sph*I fragment is described which includes the *pet* genes and three additional unidentified open reading frames. The N-terminal amino acid sequence of the isolated subunits was used for the identification of the three genes. The genes encoding the subunits are organized as follows: Rieske protein, cytochrome *b*, cytochrome c_1. Comparison of the N-terminal protein sequences with the protein sequences deduced from the nucleotide sequence showed that only cytochrome c_1 is processed during transport and assembly of the three subunits of the complex. Only the N-terminal methionine of the Rieske protein is cleaved off. The similarity of the deduced amino acid sequence of the three subunits to the corresponding subunits of other organisms is described and implications for structural features of the subunits are discussed.

Key words: Cytochrome bc_1-complex – Gene cloning – *pet* genes – Nucleotide sequence – *Rhodospirillum rubrum*

Introduction

The cytochrome bc_1-complex is a central component of photosynthetic and respiratory electron transport chains, and catalyses the oxidation of a quinol and the reduction of cytochrome *c* or plastocyanin. The complex has been isolated and characterized from various photosynthetic and respiratory organisms (Ljungdahl et al. 1987; reviewed by Hauska et al. 1983; Cramer et al. 1987; Malkin 1988). Cytochrome *bc*-complexes contain at least three functional subunits, a *b*-type cytochrome with two heme groups, a cytochrome c_1 and an iron sulfur protein known as Rieske iron sulfur protein. In some organisms, including eucaryotes additional subunits have been observed whose functional role is still unknown (Weiss et al. 1990). The existence of a cytochrome bc_1-complex in *Rs. rubrum* was first indicated by spectroscopic analysis of the chromatophore membranes (van der Wal and van Grondelle 1983) and later by isolation of the complex by Wynn et al. (1986), Kriauciunas et al. (1989) and our own group (Majewski 1989). The three-subunit complex showed the properties typical of other cytochrome *bc*-complexes.

As a prerequisite for the understanding of structure-function relationship of cytochrome *bc*-complexes the genes for the catalytic subunits of several complexes have been cloned and sequenced (reviewed by Hauska et al. 1988; Malkin 1988; Gabellini 1988). In bacteria the genes for the three catalytic subunits are arranged in a cluster named *fbc* or *pet* locus (Gabellini and Sebald 1986; Davidson and Daldal 1987; Kurowski and Ludwig 1987; Thöny-Meyer et al. 1989; Verbist et al. 1989). As bacteria are more readily accessible to genetic manipulation than eucaryotes the in vitro mutagenesis of the subunits of the complex offers the possibility to investigate the functional role of specific domains in the three subunits. Here we report the isolation and sequencing of the genes of the cytochrome bc_1-complex of *Rs. rubrum*. The three subunits were identified by comparison of the amino acid sequences deduced from the DNA sequences with the protein sequences of the N-terminal parts of the isolated subunits of a functional cytochrome bc_1-complex from *Rs. rubrum*.

Materials and methods

Enzymes and chemicals. Restriction endonucleases were from Boehringer Mannheim, FRG on Pharmacia-LKB, FRG. T7 Sequencing kit, bacterial alkaline phosphatase,

Abbreviations: BSA, bovine serum albumin; SDS, sodium dodecylsulphate; Rs, *Rhodospirillum*; Rb, *Rhodobacter*; Pc, *Paracoccus*; Rps, *Rhodopseudomonas*

The nucleotide sequence reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number X55387

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exonuclease III, S1 nuclease, phosphoramidites and pT3T7 19U were from Pharmacia-LKB, FRG. pBS SK+, EMBL3 arms and Gigapack plus were from Stratagene (Heidelberg, FRG). Helper phage R408 was obtained from Atlanta (Heidelberg, FRG). Radiochemicals were from Amersham (Braunschweig, FRG).

Bacterial strains. Rhodospirillum rubrum FR1 (DSM Nr. 1068) was obtained from the Deutsche Sammlung von Mikroorganismen (Göttingen, FRG). The strain was grown according to Omerod et al. (1961). *Escherichia coli* strains XL1-Blue, LE 392 were from Stratagene, (Heidelberg, FRG), and were grown according to standard methods (Maniatis et al. 1982).

Isolation of DNA. High molecular weight, chromosomal DNA from *Rs. rubrum* was isolated according to Silhavy et al. (1984). Plasmid DNA was obtained after amplification in the presence of chloramphenicol as described by Silhavy et al. (1984), except for the use of a step CsCl gradient according to Garger et al. (1983). Minipreparations of *E. coli* plasmids were performed according to Maniatis et al. (1982) with the alkaline lysis method. For sequencing the plasmids were further purified by chromatography on Sephacryl S-400 spun columns (Pharmacia-LKB, Freiburg). Single-stranded phagemid DNA was obtained after superinfection with the helper phage R408 following standard protocols. Lambda DNA was purified in large amounts according to Maniatis et al. (1982) and small-scale preparations were performed according to Grossberger (1987).

Construction of an EMBL3 library of Rs. rubrum. Chromosomal DNA was partially digested with restriction endonuclease *Sau*3AI. Fragments of 10–20 kb were purified by agarose gel electrophoresis and electroelution. The fragments were ligated into *Bam*HI-cleaved EMBL3 arms. Recombinant DNA was packaged with the Gigapack plus kit and used for transfection of the P2 lysogen *E. coli* P2 392. Over 90% of the library consisted of recombinant phage particles carrying a fragment of *Rs. rubrum* DNA.

Identification of positive clones. For identification of the genes for the subunits of the cytochrome bc_1-complex from *Rs. rubrum* oligonucleotides specific for conserved regions of the subunits from other organism and a heterologous probe of *Rb. capsulatus* were used. The heterologous probe was a *Pst*I fragment of pRSF1 containing most of the gene for cytochrome *b* and the gene for cytochrome c_1 of *Rhodobacter capsulatus* (kindly donated to us by W. Sebald). Plaque filter hybridization was performed according to Maniatis et al. (1982) using 5 × SSPE (20 × SSPE is: 0.2 M NaH_2PO_4 NaOH, pH 7.4, 3 M NaCl, 0.02 M EDTA), 0.5% SDS, 40% Formamide, 100 µg/ml denatured herring sperm DNA and 5 × Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA). The 2.8 kb *Pst*I fragment was labelled by oligo-primed labelling; oligonucleotides were labelled using polynucleotide kinase. After hybridization the filters were washed two times for 30 min each with 1 × SSPE, 0.1% SDS at room temperature.

Subcloning of positive fragments and DNA sequence analysis. Fragments hybridizing with the *Pst*I fragment and the oligonucleotides were subcloned into the plasmids pBS SK+ and pT3T7 19U. Nested deletions were prepared from both sides of the cloned inserts in each plasmid according to Henikoff (1987). DNA sequencing was performed by the dideoxy chain-termination procedure of Sanger et al. (1977) using [$^{35}_{\alpha}$S]dATP (600 Ci/mmol) with the T7 polymerase obtained as a kit from Pharmacia LKB. All sequencing reactions were performed with C^7dGTP.

Computer analysis of the sequences. DNA sequence analysis was performed using the program DNASIS on a personal computer (Pharmacia-LKB, Freiburg). Deduced protein sequence data were analysed using the program PC-Gene (IntelliGenetics, Genofit) at the Max-Planck Institut für Systemphysiologie in Dortmund.

Oligonucleotide synthesis. Oligonucleotides were synthesized with the Gene Assembler (Pharmacia-LKB, Freiburg, FRG) using the phosphoramidite method. Purification of the oligonucleotides was achieved using FPLC on a Mono-Q column (Pharmacia-LKB, Freiburg).

Isolation of the cytochrome bc_1*-complex and purification of the subunits.* Chromatophores were prepared from 60 g of frozen cells according to Collins and Niederman (1976). The complex was isolated from chromatophores according to Ljungdahl et al. (1987) using 0.7 mg dodecyl-β-D-maltoside per mg of protein. All buffers contained 10 mM $MgSO_4$. The subunits of the cytochrome bc_1-complex of *Rs. rubrum* were purified by SDS-polyacrylamide gel electrophoresis and electroelution. Further purification was achieved for cytochrome c_1 and the Rieske iron sulfur protein by reversed phase chromatography (RP 18.7 µ Knauer, Berlin) using a gradient of 0.1% trifluoroacetic acid in water to 0.08% trifluoroacetic acid in acetonitrile (80% in water).

N-terminal amino acid sequence analysis of the isolated subunits. The sequence was determined on an automated sequencer (Applied Biosystems) using standard Edman chemistry by Dr. H. Meyer, Institute of Physiological Chemistry, Faculty of Medicine; Ruhr-Universität, Bochum.

Results and discussion

Purification and N-terminal sequencing of the subunits of the isolated cytochrome bc_1*-complex*

The cytochrome bc_1-complex of *Rs. rubrum* was isolated using a method by Ljungdahl et al. (1987) with slight modifications. The complex consisted of three subunits as already described by Wynn et al. (1986). The activity of the complex – 350 µmole cytochrome $c_{(red.)}$/min per mg protein in the presence of 100 µg/ml dodecyl-β-D-maltoside – was one of the highest reported for bacterial cytochrome *bc*-complexes. The three subunits were iso-

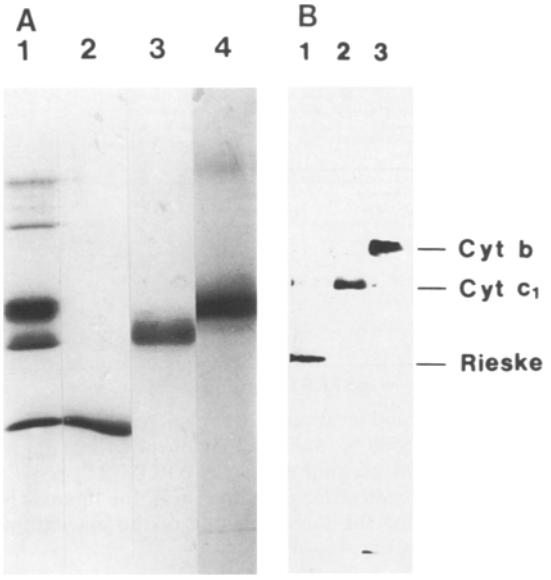

Fig. 1. A SDS-PAGE of the cytochrome bc_1-complex and the isolated subunits of the complex from *Rs. rubrum*. Five micrograms of each subunit and 12 µg of the complex were loaded onto a 15% polyacrylamide gel according to Schägger et al. (1987). The gel was stained with Coomassie brillant blue. Lane 1, purified bc_1 complex; lane 2, Rieske protein; lane 3, cytochrome c_1 lane 4, cytochrome *b*. **B** Western blot of the purified cytochrome bc_1-complex of *Rs. rubrum*. Nine micrograms of complex were loaded per lane. The purified antibodies were diluted 250-fold. Lane 1, anti-Rieske protein, serum 1; lane 2, anti-cytochrome c_1, serum 1; lane 3, anti-cytochrome *b*, serum 2

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1. Rieske FeS-protein
       1        10        20        30
RR:    AEAEHTASTP GGESSRRDFL IYGTTAVGAV GVAL
PD:  M SHADEHAGDH GA--TRRDFL YYATAGAGTV AAGA
RC:  M SHAEDNAGT- -----RRDFL YHATAATGVV VTGA
RV:  M ASSDTAEAT- -----RRDFL YVATAAVGAA GVAA
                      *****    *   *

2. Cytochrome b
       1        10        20        30
RR:    MYTPPRWNNK --ALKWFDER LPVL-----T VAHE
PD: MA GIPHDHYEPK TGFERWLHRR LPIVSLVYDT LMIP
RC: MS GIPHDHYEPK TGIEKWLHDR LPIVGLYYDT IMIP
RV: MS G--HSSYQPS TGIERWLDTR LPIVRMMYDQ FVAF
                  *   * **

3. Cytochrome c1
       1        10        20
RR:    NEGGVSLHKQ DWSWKGIFGR YDQPQL
PD:    SHAAAHIEDI SFSFEGPFGK FDQHQL
RC:       NSNVQDH AFSFEGIFGK FDQAQL
RV:     SGGDTPHLQ SWSFAGPFGQ YDKAQL
                  *  * **   ** **
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Fig. 2. Comparison of the N-terminal sequences of the isolated protein subunits of the cytochrome bc_1-complex from *Rs. rubrum* with the corresponding proteins of *Rb. capsulatus*, RC (Gabellini and Sebald 1986; Davidson and Daldal 1987) *Pc. denitrificans*, PD (Kurowski and Ludwig 1987) and *Rps. viridis*, RV (Verbist et al. 1989). The amino acids are shown in the single letter code. Identities are marked by an *asterisk* below the sequences

Table 1. Oligonucleotides coding for conserved regions of the Rieske protein, cytochrome *b* and cytochrome c_1 from different organisms. For synthesis of the oligonucleotides the preferred codon usage of *Rs. rubrum* genes, with a discrimination against A and T in the third position, was applied

1. Cytochrom b No.1	5'-TGG	GGN	CAR	ATG	TCS	TTY	TGG	GG-3'
	Trp	Gly	Gln	Met	Ser	Phe	Trp	Gly
2. Cytochrom b No.2	5'-ATY	GTB	CCB	GAR	TGG	TAY	TTY	CT-3'
	Ile	Val	Pro	Glu	Trp	Tyr	Phe	Leu
3. Cytochrom c_1	5'-TGG	TTY	TGY	CCS	TGY	CAY	GG-3'	
	Trp	Phe	Cys	Pro	Cys	His	Gly	
4. Rieske protein	5'-GAR	GTB	TGY	TCS	GCR	TGY	CA-3'	
	Glu	Val	Cys	Ser	Ala	Cys		

R – A or G; S – C or G; Y – C or T; B – C, G or T; N – A, C, G or T

bald 1986) but was later identified as the genes from *Rb. capsulatus* (Daldal et al. 1989), and four synthetic oligonucleotides specific for conserved regions of known sequence in the homologous proteins from other organisms (see Table 1). A genomic library of *Rs. rubrum* DNA was established in the replacement phage EMBL3, consisting of 5×10^5 independent clones with a mean insert size of between 10 and 20 kb. Seven clones were isolated which hybridized to both the 2.8 kb fragment of *Rb. capsulatus* and the oligonucleotide number 3 (Table 1). By restriction mapping these clones could be classified into four groups. Two of these groups contained only short segments of the genes for the cytochrome

lated by preparative SDS-gel electrophoresis (Fig. 1) and were shown by reversed phase chromatography to be contaminated only by minor impurities. Using specific antibodies against the isolated subunits the faint bands in the high molecular weight region of the gel were identified as aggregates of the different subunits (data not shown). These antibodies were raised in two rabbits each against each of the isolated polypeptides of the complex. In chromatophore membranes these antibodies reacted only with the subunit used as immunogen. In the highly purified complex one of the two cytochrome *b* antibodies cross-reacted slightly with the cytochrome c_1 subunit. There was little cross-reaction of these antibodies with the homologous subunits from beef or yeast mitochondria.

The N-terminal amino acid sequence was determined by automated Edman degradation of the isolated subunits. The sequence was determined for the first 27 amino acids of cytochrome *b*, 34 residues of the Rieske protein and 26 amino acids of cytochrome c_1 as shown in Fig. 2. The subunits show weak but significant homology to the N-terminal parts of the proteins of *Rb. capsulatus*, *Rps. viridis* and *Paracoccus denitrificans*.

Identification of the genes

For the identification of the *petABC* genes of *Rs. rubrum* we used a heterologous probe (kindly provided by W. Sebald; Gabellini et al. 1985), which was originally thought to be from *Rb. sphaeroides* (Gabellini and Se-

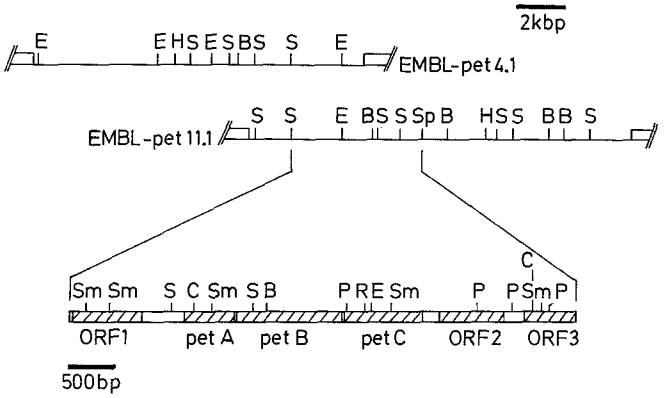


Fig. 3. Restriction map of the phages EMBL-*pet* 4.1 and EMBL-*pet* 11.1 which carry the genes for the three subunits of the cytochrome bc_1-complex of *Rs. rubrum*. The 5500 bp *SalI*/*SphI* fragment of EMBL-*pet* 11.1 was subcloned into three different plasmid vectors. The location of open reading frames on this fragment is indicated by the *shaded regions*. The size of the cloned fragments is indicated. The arms of the EMBL phages are not shown. Abbreviations of restriction endonucleases: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; P, *Pvu*I; S, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; R, *Pst*I

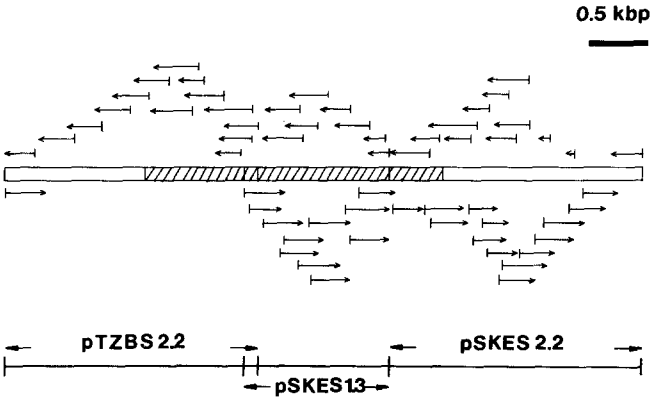


Fig. 4. Size, orientation and location of the deletion subclones used for sequencing of the 5500 bp *SphI*/*SalI* fragment of EMBL-*pet* 11.1. The three different plasmid subclones pTZBS 2.2, pSKES 1.3 and pSKES 2.2 are indicated at the bottom of the figure. The location of the genes for the three subunits of the cytochrome bc_1-complex is shown by the *shaded area*

bc_1-complex. The clones EMBL-*pet* 11.1 and EMBL-*pet* 4.1 were further characterized. A crude restriction map giving the localization of the *pet* genes on the cloned fragment is shown in Fig. 3. The genes coding for the Rieske protein (*petA*), cytochrome *b* (*petB*) and cytochrome c_1 (*petC*) were identified by hybridization with the specific oligonucleotides described above and subcloned as a 2.2 kb *SphI* – *Bam*HI fragment and a 1.15 kb and 2.2 kb *Eco*RI – *Sal*I fragment.

Nucleotide sequencing strategy

The complete sequence of 5550 bp of the clone EMBL3-*pet* 11.1 carrying the *pet* genes of *Rs. rubrum* was determined. Ordered deletions of the three subclones were prepared by exonuclease III digestion from both sides of the cloned inserts, as described in Materials and methods. Overlapping clones were sequenced in one direction as single-stranded phagemid DNA and in the other direction as closed circular plasmid DNA. The locations of the *pet* genes and the sequenced deletion clones are shown in Fig. 4.

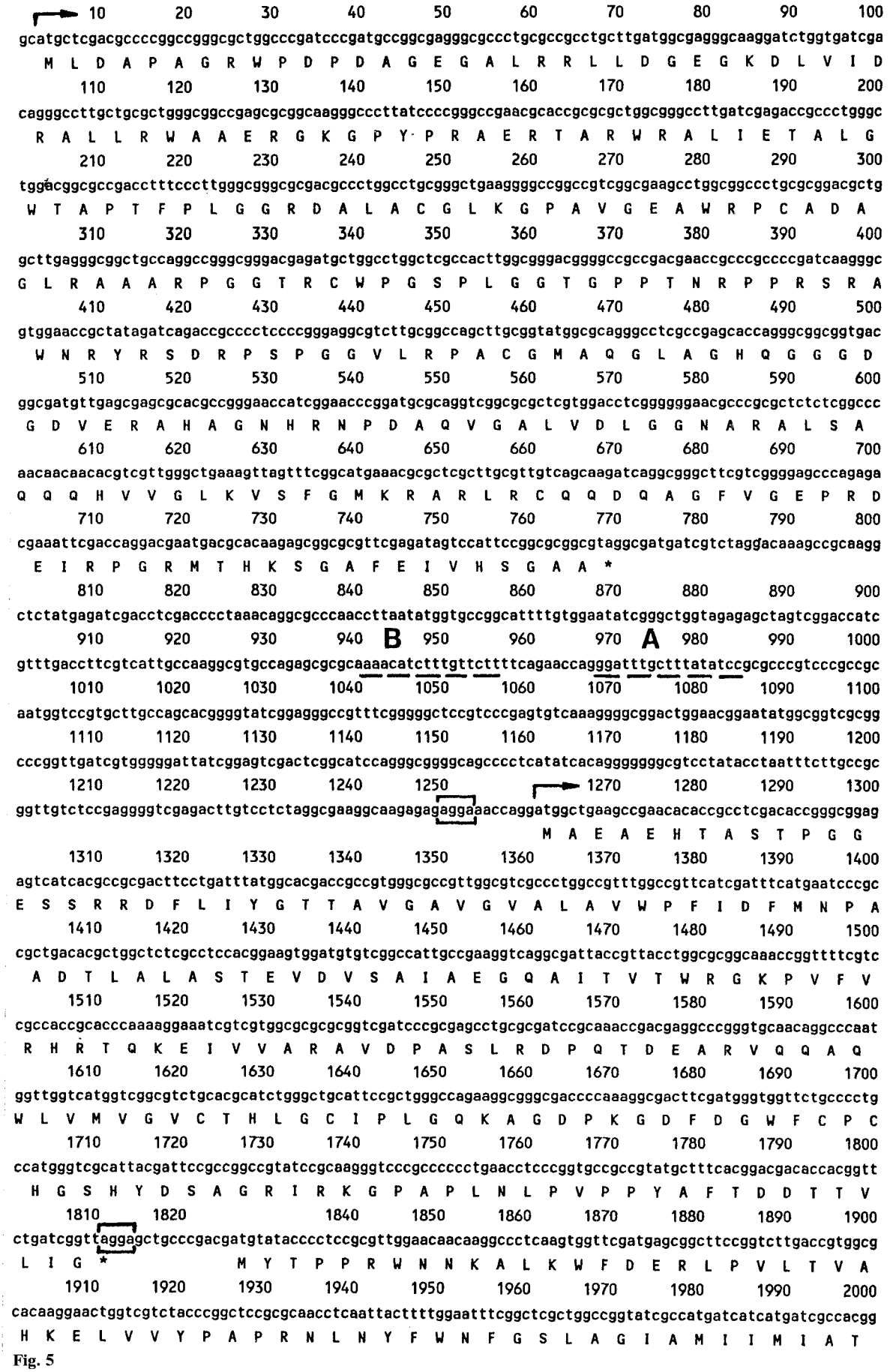
Nucleotide sequence of the 5500 bp SalI – SphI *fragment*

The nucleotide sequence of the *pet* genes was obtained for both strands except for the major part of the Rieske protein which was only sequenced on one strand. The sequence obtained for the whole 5550 bp fragment and the deduced amino acid sequence of the three subunits and the other open reading frames (ORFs) in this region are shown in Fig. 5. The ORFs for the *pet* genes were identified by comparison with the N-terminal amino acid sequence of the isolated subunits (see Fig. 2) and by their homology to the polypeptides of *Rb. capsulatus* (Gabellini and Sebald 1986; Davidson and Daldal 1987).

The three subunits were coded in the following order: Rieske protein (*petA*), cytochrome *b* (*petB*) and cytochrome c_1 (*petC*). The gene for the Rieske protein starts with an ATG codon at nucleotide 1261 and ends at the TAG codon at position 1810. The gene is preceded by a typical Shine-Dalgarno sequence seven nucleotides upstream of the ATG start codon. This sequence is complementary to the 16S ribosomal RNA of *Rs. rubrum* (Gibson et al. 1979). The gene for cytochrome *b* starts at codon 1826 and is separated from *petA* by 13 nucleotides. The Shine-Dalgarno sequence of *petB* is built from part of the stop codon of *petA* and is located 12 nucleotides upstream of the start codon of *petB*. The gene ends with a TGA stop codon at nucleotide 3041 and is separated from the start codon of cytochrome c_1 at position 3070 by 26 nucleotides. A TAA stop codon ends the gene for cytochrome c_1 at nucleotide 3886. The gene is preceded by a typical Shine-Dalgarno sequence at a distance of 10 nucleotides from the ATG codon. There is an additional in-frame ATG start codon at nucleotide 2682 which would lead to a protein with 401 amino acid residues. This start codon has probably no functional role in vivo.

About 200 bp upstream of the *pet* operon there are regions homologous to the promoter sequences of the gene for Rubisco (Leustek et al. 1988) and the *ATP*

Fig. 5. Nucleotide sequence of the 5500 bp *SalI*/*SphI* fragment of EMBL-*pet* 11.1 and derived amino acid sequences of the Rieske FeS-protein (*petA*), cytochrome *b* (*petB*) and cytochrome c_1 (*petC*) and the flanking reading frames. Start codons are indicated by *arrows*, stop codons are indicated by *asterisks*. The *underlined* regions resemble the structures with dyad symmetry (terminator 1, −29 kcal; terminator 2, −17 kcal). The regions indicated with a *dashed line* correspond to the sequence similar to other *Rs. rubrum* promotor sequences. The presumed Shine-Dalgarno sequences are *boxed*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gcatgctcgacgccccggccgggcgctggcccgatcccgatgccggcgagggcgccctgcgccgcctgcttgatggcgagggcaaggatctggtgatcga
M L D A P A G R W P D P D A G E G A L R R L L D G E G K D L V I D
110 120 130 140 150 160 170 180 190 200
cagggccttgctgcgctgggcggccgagcgcggcaagggcccttatccccgggccgaacgcaccgcgcgctggcgggccttgatcgagaccgccctgggc
R A L L R W A A E R G K G P Y P R A E R T A R W R A L I E T A L G
210 220 230 240 250 260 270 280 290 300
tggacggcgccgacctttcccttgggcgggcgcgacgccctggcctgcgggctgaaggggccggccgtcggcgaagcctggcggccctgcgcggacgctg
W T A P T F P L G G R D A L A C G L K G P A V G E A W R P C A D A
310 320 330 340 350 360 370 380 390 400
gcttgagggcggctgccaggccgggcgggacgagatgctggcctggctcgccacttggcgggacggggccgccgacgaaccgcccgccccgatcaagggc
G L R A A A R P G G T R C W P G S P L G G T G P P T N R P P R S R A
410 420 430 440 450 460 470 480 490 500
gtggaaccgctatagatcagaccgcccctccccgggaggcgtcttgcggccagcttgcggtatggcgcagggcctcgccgagcaccagggcggcggtgac
W N R Y R S D R P S P G G V L R P A C G M A Q G L A G H Q G G G D
510 520 530 540 550 560 570 580 590 600
ggcgatgttgagcgagcgcacgccgggaaccatcggaacccggatgcgcaggtcggcgctcgtggacctcgggggaacgcccgcgctctctcggccc
G D V E R A H A G N H R N P D A Q V G A L V D L G G N A R A L S A
610 620 630 640 650 660 670 680 690 700
aacaacaacacgtcgttgggctgaaagttagtttcggcatgaaacgcgctcgcttgcgttgtcagcaagatcaggcgggcttcgtcggggagcccagaga
Q Q Q H V V G L K V S F G M K R A R L R C Q Q D Q A G F V G E P R D
710 720 730 740 750 760 770 780 790 800
cgaaattcgaccaggacgaatgacgcacaagagcggcgcgttcgagatagtccattccggcgcggcgtaggcgatgatcgtctaggacaaagccgcaagg
E I R P G R M T H K S G A F E I V H S G A A *
810 820 830 840 850 860 870 880 890 900
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1110 1120 1130 1140 1150 1160 1170 1180 1190 1200
cccggttgatcgtgggggattatcggagtcgactcggcatccagggcggggcagcccctcatatcacagggggggcgtcctatacctaatttcttgccgc
1210 1220 1230 1240 1250 1270 1280 1290 1300
ggttgtctccgaggggtcgagacttgtcctctaggcgaaggcaagagaggaggaaaccaggatggctgaagccgaacacaccgcctcgacaccgggcggag
M A E A E H T A S T P G G
1310 1320 1330 1340 1350 1360 1370 1380 1390 1400
agtcatcacgccgcgacttcctgatttatggcacgaccgccgtgggcgccgttggcgtcgccctggccgtttggccgttcatcgatttcatgaatcccgc
E S S R R D F L I Y G T T A V G A V G V A L A V W P F I D F M N P A
1410 1420 1430 1440 1450 1460 1470 1480 1490 1500
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A D T L A L A S T E V D V S A I A E G Q A I T V T W R G K P V F V
1510 1520 1530 1540 1550 1560 1570 1580 1590 1600
cgccaccgcacccaaaaggaaatcgtcgtggcgcgcgcggtcgatcccgcgagcctgcgcgatccgcaaaccgacgaggcccgggtgcaacaggcccaat
R H R T Q K E I V V A R A V D P A S L R D P Q T D E A R V Q Q A Q
1610 1620 1630 1640 1650 1660 1670 1680 1690 1700
ggttggtcatggtcggcgtctgcacgcatctgggctgcattccgctgggccagaaggcgggcgaccccaaaggcgacttcgatgggtggttctgcccctg
W L V M V G V C T H L G C I P L G Q K A G D P K G D F D G W F C P C
1710 1720 1730 1740 1750 1760 1770 1780 1790 1800
ccatgggtcgcattacgattccgccggccgtatccgcaagggtcccgccccctgaacctcccggtgccgccgtatgctttcacggacgacaccacggtt
H G S H Y D S A G R I R K G P A P L N L P V P P Y A F T D D T T V
1810 1820 1840 1850 1860 1870 1880 1890 1900
ctgatcggttaggagctgcccgacgatgtataccctccgcgttggaacaacaaggccctcaagtggttcgatgagcggcttccggtcttgaccgtggcg
L I G * M Y T P P R W N N K A L K W F D E R L P V L T V A
1910 1920 1930 1940 1950 1960 1970 1980 1990 2000
cacaaggaactggtcgtctacccggctccgcgcaacctcaattacttttggaatttcggctcgctggccggtatcgccatgatcatcatgatcgccacgg
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Fig. 5

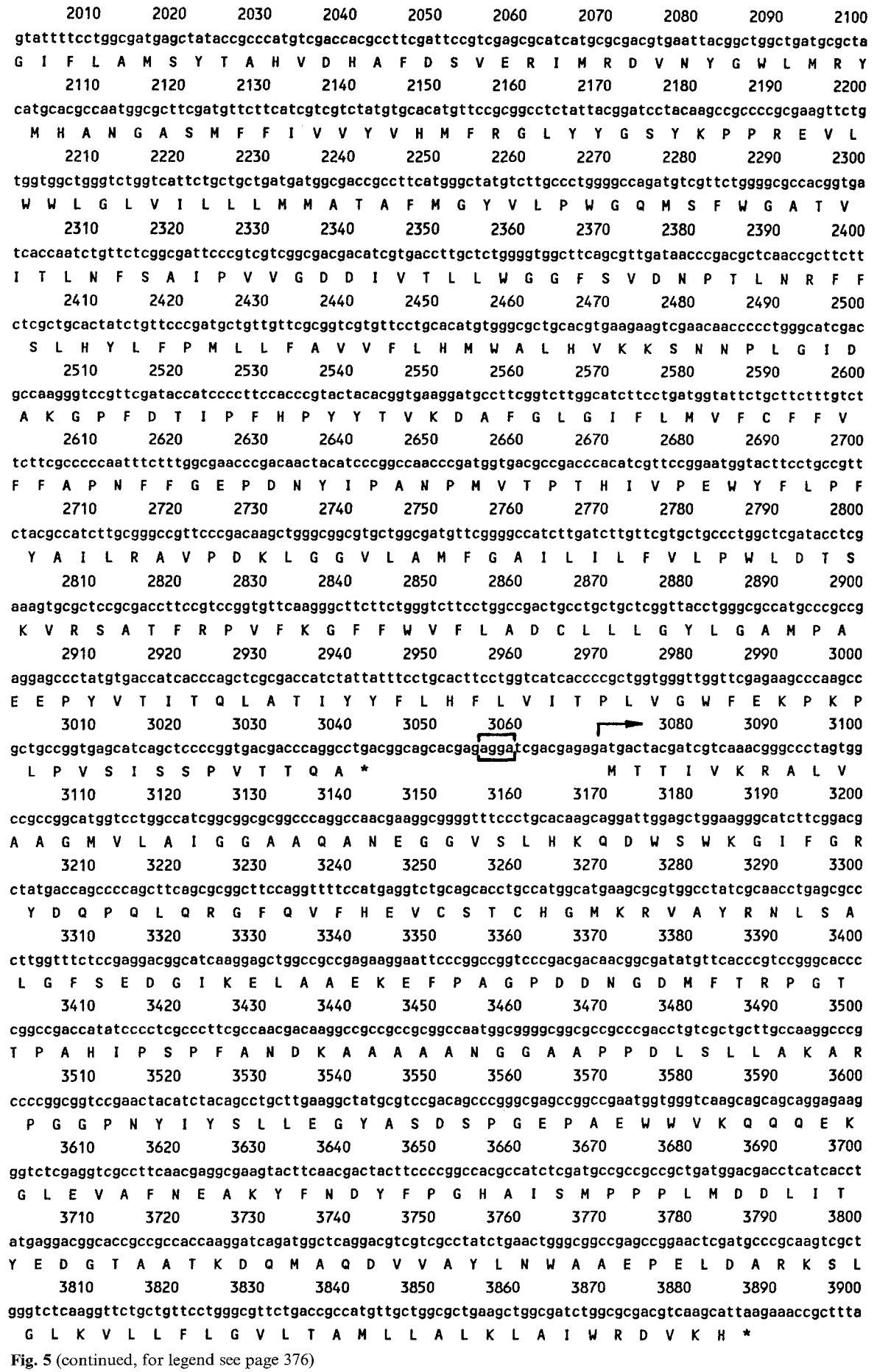

Fig. 5 (continued, for legend see page 376)

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     4010       4020       4030       4040       4050       4060                  4080       4090       4100
tccgggggggtggcgcgctaaggtgccccacccgcaaaagggtgagccagccaggaagaggggaagacatgtccgaggcgtatcggcagccggtgcttgg
                                                                                 M  S  E  A  Y  R  Q  P  V  L  G
     4110       4120       4130       4140       4150       4160       4170       4180       4190       4200
ggtcatcggcggatcgggggtttatgatatcgacggtctggaaggggcgcgttggcaaacggtggaaagcccgttcggcgacgtttccgatcagatcctg
  V  I  G  G  S  G  V  Y  D  I  D  G  L  E  G  A  R  W  Q  T  V  E  S  P  F  G  D  V  S  D  Q  I  L
     4210       4220       4230       4240       4250       4260       4270       4280       4290       4300
cgcggcaccctggatgggctggagatggcctttctgccccgccatggccgtggccatgtgctcgccccctccgatgtgaactaccgcgccaatatcgacg
  R  G  T  L  D  G  L  E  M  A  F  L  P  R  H  G  R  G  H  V  L  A  P  S  D  V  N  Y  R  A  N  I  D
     4310       4320       4330       4340       4350       4360       4370       4380       4390       4400
ccctgaagcgggcgggcgtgaccgagatcttgtcggtctccgccgtcggttctctggccgaggacctgccgccgggcaccttcgtcatcgccgatcagtt
  A  L  K  R  A  G  V  T  E  I  L  S  V  S  A  V  G  S  L  A  E  D  L  P  P  G  T  F  V  I  A  D  Q  F
     4410       4420       4430       4440       4450       4460       4470       4480       4490       4500
catcgaccgcaccttcgcccgcgagaagagctttttttcggcaagggtctggtcgcccatgtcagcatggcccatccggtcagcgcctggctgggcgatcg
  I  D  R  T  F  A  R  E  K  S  F  F  R  Q  G  S  G  R  P  C  Q  H  G  P  S  G  Q  R  L  A  G  R  S
     4510       4520       4530       4540       4550       4560       4570       4580       4590       4600
cgtcgaagaggttctggccgatctggccattccccatcgccggggcggcacctatctgtgcatggaggggccgcagttctcgaccctggccgaaagcaat
  R  R  R  G  S  G  R  S  G  H  S  P  S  P  G  R  H  L  S  V  H  G  G  A  A  V  L  D  P  G  R  K  Q
     4610       4620       4630       4640       4650       4660       4670       4680       4690       4700
ctctatcggcaatggggctgccacgtcatcggcatgaccaacatgcccgaggccaagctggcccgcgaagccgagatcgcctattgcaccgtggccatgg
  S  L  S  A  M  G  L  P  R  H  R  H  D  Q  H  A  R  G  Q  A  G  P  R  S  R  D  R  L  L  H  R  G  H  G
     4710       4720       4730       4740       4750       4760       4770       4780       4790       4800
tcaccgatttcgactgctggcaccccgatcacgaccacgtcagcgtcgaggcggtggttcgcgtgctgctgcaaaacgccgataaggcccgctccctggt
  H  R  F  R  L  L  A  P  R  S  R  P  R  Q  R  R  G  G  G  S  R  A  A  A  K  R  R  *
     4810       4820       4830       4840       4850       4860       4870       4880       4890       4900
caaggcgatgcccgccaagctcaaggaccggccctatcccttgcccgatggcagccaccgcagcctggacaacgccatcatcacccatcccgatcgccgc
     4910       4920       4930       4940       4950       4960       4970       4980       4990
aaccccgggatggcgcgcaaattgagcgccgtcgccggtcgggtgctgggctaatccaccccgcttgggggcggtttttacaaggatgatcggccgatga
                                                                                                 M
     5010       5020       5030       5040       5050       5060       5070       5080       5090       5100
acgtcaaaggcactcctacccgtacgatctggccggcccgcgaaggcggggcggtgtggatcatcgatcaaacccggcttccccacgagttcgtcaccca
  N  V  K  G  T  P  T  R  T  I  W  P  A  R  E  G  G  A  V  E  I  I  D  Q  T  R  L  P  H  E  F  V  T  Q
     5110       5120       5130       5140       5150       5160       5170       5180       5190       5200
gcgcctgaacgacctgggcgccgtggcccatgccatccgcgccatgctggtgcgcggcgccccgctgatcggggcgaccgctgcctatggcgtggcctta
  R  L  N  D  L  G  A  V  A  H  A  I  R  A  M  L  V  R  G  A  P  L  I  G  A  T  A  A  Y  G  V  A  L
     5210       5220       5230       5240       5250       5260       5270       5280       5290       5300
ggcatggccgaggacccgagcgacgaggggttgacccgggcttgtcaaaccctgctcgccacccggccgacggcggtcaatctgcgctgggcgatcgagg
  G  M  A  E  D  P  S  D  E  G  L  T  R  A  C  Q  T  L  L  A  T  R  P  T  A  V  N  L  R  W  A  I  E
     5310       5320       5330       5340       5350       5360       5370       5380       5390       5400
cgatggccgagtctctggcggccgtgccccccgaccagcgggcgcaagcggcctgggccaaggccggggcgatctgtgacgaggacgtggcgctgaacga
  A  M  A  E  S  L  A  A  V  P  P  D  Q  R  A  Q  A  A  W  A  K  A  G  A  I  C  D  E  D  V  A  L  N  E
     5410       5420       5430       5440       5450       5460       5470       5480       5490       5500
ggccatcggcgaccatggcctggggatcattaaggatctggcccggaccaagggcgtggaaaagggcggcgagggcccgatcaatatcctgacgcattgc
  A  I  G  D  H  G  L  G  I  I  K  D  L  A  R  T  K  G  V  E  K  G  G  E  G  P  I  N  I  L  T  H  C
     5510       5520       5530       5540       5550
aacgcaggctggctagccaccgtcgattgggcacggccctggcgccggct
  N  A  G  W  L  A  T  V  D  W  A  R  P  W  R  R
```

Fig. 5 (continued)

operon (Falk et al. 1985) of *Rs. rubrum*. At positions 3917 and 4005 two regions of strong dyad symmetry were found. The first one resembles in its properties and high stability the rho-independent transcription terminators in *E. coli*. Similar structures have also been observed in the operon of *Rb. capsulatus* (Gabellini and Sebald 1986). These authors could show by transcript mapping that probably only the second termination structure is used in vivo.

There were three additional ORFs on the coding strand (see Fig. 3). The first ORF starts with the second nucleotide of the isolated fragment, the other two ORFs

are preceded by a typical Shine-Dalgarno sequence. There was no similarity of the protein sequences to previously published sequences of the additional subunits of the cytochrome bc_1-complex from the mitochondria of eucaryotes.

Amino acid sequence of the genes

Rieske FeS protein. The gene for the Rieske protein codes for a polypeptide with 183 amino acids and an apparent molecular weight of 19512. In our preparations of the cytochrome bc_1-complex we observed a molecular weight of 23000 for the mature Rieske protein. Comparison of the N-terminal sequence of the isolated subunit with the deduced amino acid sequence showed that only the N-terminal methionine had been cleaved off during assembly of the complex. Thus the molecular weight observed by gel electrophoresis is an overestimate as was also reported for the proteins of *Rb. capsulatus* (Gabellini and Sebald 1986) and *Neurospora crassa* (Harnisch et al. 1985).

The deduced amino acid sequence of the Rieske iron sulfur protein shows 57% similarity to that of the protein of *Rps. viridis*, 51% similarity to the protein in *Rb. capsulatus* and 48% to the protein of *Paracoccus denitrificans* (Kurowski and Ludwig 1987). Comparison with mitochondrial proteins gave values of between 45% and 50% homology, whereas comparison to plastidial and cyanobacterial proteins yielded a similarity of between 18% and 20%. The C-terminal-region (119 GVCTHLGCIPLGQKA 133 and 138 GDFDGWFCPCHGSHYD 153), which is probably involved in binding of the FeS cluster, is highly conserved. However four additional amino acids (GDPK) between these two domains are only observed in the protein from *Rs. rubrum*. The conserved glycine in position 141 is changed to aspartate.

A hydropathy analysis of the Rieske protein is shown in Fig. 6A. This analysis confirms the structure already described for other Rieske proteins. There is one hydrophobic stretch anchoring the Rieske protein to the membrane. This domain, possibly helical, is bordered by two negatively charged aspartates (residues 19 and 42). It is not known whether the protein is anchored to the membrane by one or two helices as proposed by Hartl et al. (1986) or whether the protein is only associated with the membrane as indicated by the very long hydrophobic region. The major part of the protein is hydrophilic and is exposed to the aqueous environment. The region between residues 65 and 105 contains a large number of charged residues. The positively charged residues between glutamate 67 and lysine 86 could be involved in binding of the negatively charged cytochrome c_1.

Cytochrome b. The gene for cytochrome *b* codes for a protein of 405 amino acid residues corresponding to an apparent molecular weight of 46366. In SDS gels a molecular weight of 37000 was calculated from the relative mobility of the cytochrome *b* subunit in the isolated

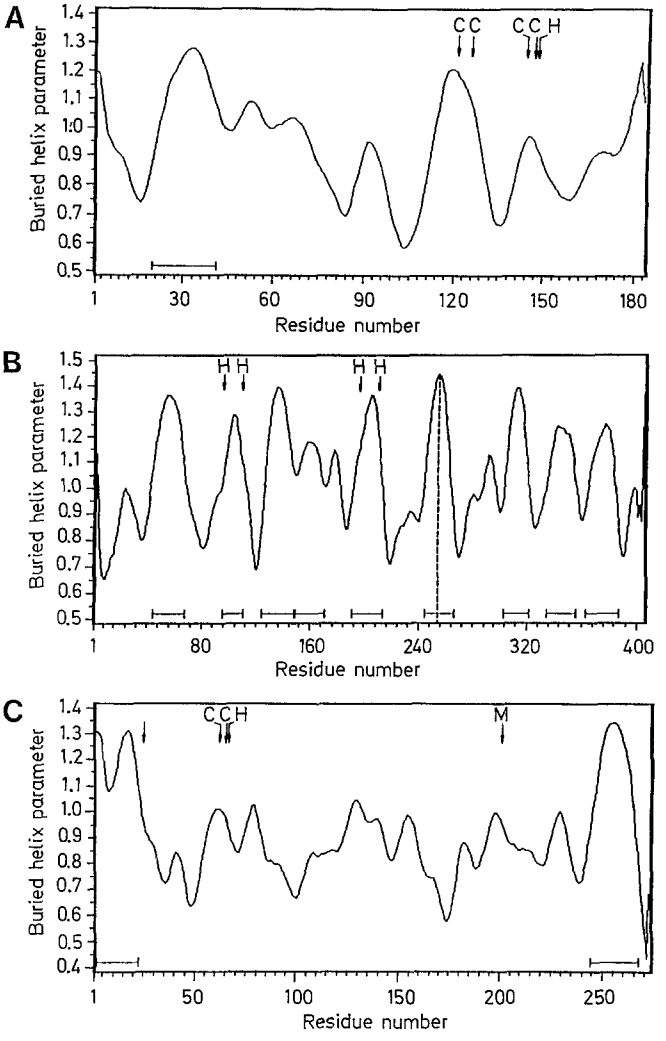

Fig. 6A–C. The Rao-Argos algorithm of the program package PC-Gene from IntelliGenetics was used for calculation of the hydropathy profiles. Probable membrane-spanning helices are indicated by the *bar* below the profile. **A** Hydropathy profile of the Rieske-protein from *Rs. rubrum*. The positions of the four cysteines (C) and the histidines (H) which are involved in binding of the FeS-cluster are indicated by *arrows*. **B** Hydropathy profile of cytochrome *b* from *Rs. rubrum*. The positions of the four histidines (H) which are involved in binding of the two heme groups are indicated by *arrows*. **C** Hydropathy profile of cytochrome c_1 from *Rs. rubrum*. The positions of the two cysteines (C), the histidine (H) and methionine (M) which are involved in binding of the heme group are indicated by *arrows*. The position of the cleavage site of the leader peptidase is also indicated by an arrow

cytochrome bc_1-complex. This discrepancy between the actual molecular weight and the observed value seems to be common to other cytochrome *b* subunits isolated (Gabellini and Sebald 1986), and is possibly due to increased binding of SDS to the very hydrophobic protein.

Cytochrome *b* possess 61% similarity to the protein of *Rps. viridis*. The degree of similarity to the corresponding proteins of *Rb. capsulatus* and *Pc. denitrificans* (Davidson and Daldal 1987; Kurowski and Ludwig 1987) is 53%; inclusion of conservative substitutions increases this value to 83%. The protein shows 54% homology to cytochrome *b* of plant mitochondria, 46%

similarity to that of animal mitochondria and 26% to cytochrome *b* and subunit IV of plants and cyanobacteria. The regions of residues 58–200, 262–298 and 305–330 are almost entirely conserved. These regions also contain the domains in which the four histidines involved in heme binding are located. The four histidines and the positively charged residues R91, R111, R190 and H214 are entirely conserved as already described for other cytochromes *b* (Saraste 1984; Widger et al. 1984). There are two gaps in cytochrome *b* of *Rs. rubrum* compared to the proteins of *Rhodobacter* or *Paracoccus*, between residues K227 and G228 (6 residues) and A298 and V299 (18 residues). These gaps were also observed for the mitochondrial cytochrome *b*. In this regard the protein of *Rs. rubrum* is more homologous to the mitochondrial cytochrome *b* than to its bacterial counterparts.

Cytochrome *b* is a very hydrophobic protein. A hydropathy analysis of cytochrome *b* with the algorithm of Rao-Argos (Argos et al. 1982) shows eight to nine trans-membrane helices (Fig. 6B). The analysis of mutations conferring resistance to myxothiazol and antimycin has shown that helix four is not in the membrane (Howell and Gilbert 1988; di Rago and Colson 1988; di Rago et al. 1989; Daldal et al. 1989). Thus an eight helix model of cytochrome *b* is more generally accepted (Crofts et al. 1987; Brasseur 1988). This is confirmed by an analysis of cytochrome *b* from *Rs. rubrum* according to Eisenberg et al. (1984) which predicts a membrane surface-associated location for helix four.

Cytochrome c_1. The *petC* gene codes for 272 residues with an apparent molecular weight of 29493. From the electrophoretic mobility of the cytochrome c_1 subunit in the isolated complex a molecular weight of 30000 was calculated. As will be discussed later cytochrome c_1 possesses a signal sequence, which is cleaved off in the mature protein yielding a size of 27500 for the processed subunit.

Cytochrome c_1 is the least conserved protein of the three subunits of the cytochrome bc_1-complex. The protein shows a similarity of 41% to cytochrome c_1 of *Rps. viridis*. The similarity to the proteins of *Rhodobacter* and *Paracoccus* is only 33%, the similarity to mitochondrial proteins is 38% and to plant-type cytochrome *f* only 13%. Significant similarity is restricted to the regions of heme binding from residues 38 to 80, a short domain between P135 and P145 and the C-terminal part of the protein. According to a hydropathy analysis this latter part forms a hydrophobic helix which anchors the protein to the membrane (Fig. 6C). Another hydrophobic helix is located at the N-terminal part of the protein and is part of the signal sequence which is cleaved off after maturation of this subunit. A methionine is discussed as the sixth ligand of the heme group (Tervoort and van Gelder 1983). This methionine could be residue 200 which is conserved in all cytochromes c_1 except the plant-type cytochrome *f*. In these proteins the sixth ligand is probably a lysine residue (Davis et al. 1988). The protein possesses two regions with a large number of negatively charged residues. These are the domain from residues 205 to 215 which is highly conserved and contains four negative charges, and the domain from residues 81 to 104 which is very poorly conserved. Nevertheless this region contains seven negative charges which are also observed in other cytochromes c_1.

Signal sequences. The amino acid sequences as derived from the nucleotide sequences of the genes for the three subunits were compared with the N-terminal sequences of the mature subunits in the isolated complex. There is no processing of cytochrome *b* as the N-terminal methionine is contained in the mature protein. From the Rieske protein the N-terminal methionine is cleaved off but there is no indication of a signal sequence as proposed by Gabellini and Sebald (1986). Cytochrome c_1 is processed to a mature form with a molecular weight of 27500 by removing a N-terminal leader sequence of 24 amino acid residues. During the first cycle of the Edman degradation two amino acid residues, aparagine and glutamate, were detected in a ratio of 98% to 2% respectively. As glutamate is the second residue after the assumed cleavage site of the leader peptidase this heterogeneity probably indicates a small inaccuracy in processing. The properties of the leader peptide were similar to those of typical bacterial and eucaryotic signal sequences (Gierasch 1989; Randall and Hardy 1989) which a positively charged region at the extreme N-terminus followed by a hydrophobic sequence.

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