Cloning and expression of the fbc operon encoding the FeS protein, cytochrome b and cytochrome c₁ from the Rhodopseudomonas sphaeroides b/c₁ complex


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The gene for the FeS protein of the Rhodopseudomonas sphaeroides b/c₁ complex was identified by means of cross-hybridization with a segment of the gene encoding the corresponding FeS protein of Neurospora crassa. Plasmids (pRSF1-14) containing the cross-hybridizing region, covering in total 13.5 kb of chromosomal DNA, were expressed in vitro in a homologous system. One RSF plasmid directed the synthesis of all three main polypeptides of the R. sphaeroides b/c₁ complex: the FeS protein, cytochrome b and cytochrome c₁. The FeS protein and cytochrome c₁ were apparently synthesized as precursor forms. None of the pRSF plasmids directed the synthesis of the 10-kd polypeptide found in b/c₁ complex preparations. Partial sequencing of the cloned region was performed. Several sites of strong homology between R. sphaeroides and eukaryotic polypeptides of the b/c₁ complex were identified. The genes encode the three b/c₁ polypeptides in the order: (5') FeS protein, cytochrome b, cytochrome c₁. The three genes are transcribed to give a polycistronic mRNA of 2.9 kb. This transcriptional unit has been designated the fbc operon; its coding capacity corresponds to the size of the polycistronic mRNA assuming that only the genes for the FeS protein (b/c₁F), cytochrome b (b/c₁B) and cytochrome c₁ (b/c₁C) are present. This could indicate that these three subunits constitute the minimal catalytic unit of the b/c₁ complex from photosynthetic membranes.

Key words: R. sphaeroides b/c₁ complex/gene cloning/in vitro expression/polygenic RNA

Introduction

The electron transport chain of the phototrophic bacterium Rhodopseudomonas sphaeroides comprises a ubiquinol cytochrome c oxidoreductase or b/c₁ complex that catalyses cyclic photosynthetic electron transport together with the photochemical reaction center, ubiquinol and a soluble cytochrome c₅ (Dutton and Prince, 1978). In aerobic growth conditions the b/c₁ complex of R. sphaeroides functions in a respiratory chain of mitochondrial type (Baccarini Melandri and Zannoni, 1978). The b/c₁ complex isolated in its active form from photosynthetic membranes of R. sphaeroides GA (Gabellini et al., 1982) and R. sphaeroides R26 (Yu and Yu, 1982), comprises four polypeptides: cytochrome b (40 kD), cytochrome c₁ (33 kD), FeS protein (24 kD) and a 10-kD polypeptide (Hauska et al., 1983). Proton translocation activity and membrane potential generation are reconstituted when the enzyme is incorporated into liposomes (Hurt et al., 1983). Although it has a simpler composition, this bacterial b/c₁ complex shows many functional similarities to the corresponding mitochondrial complex. The substrate specificity, sensitivity to inhibitors, spectral characteristics and redox properties are largely comparable (Gabellini et al., 1982; Gabellini and Hauska, 1983a), suggesting that the essential features of the enzyme structure have been highly conserved. Whilst there have been recent advances in the biochemical characterization and kinetic analysis of the b/c₁ complex of R. sphaeroides (Crofts et al., 1983), there has been no information up to now on the primary structure of this complex. Here we describe the initial steps towards a complete description of the sequence and mode of expression of the genes encoding the b/c₁ complex subunits of R. sphaeroides.

The gene encoding the Rieske FeS protein (Rieske, 1964) of Neurospora crassa has been cloned recently and sequenced (Harnisch et al., in preparation). The observation that antibodies raised against the Rieske FeS protein of N. crassa cross-reacted with the FeS protein of R. sphaeroides b/c₁ complex (Gabellini and Hauska, unpublished) suggested the use of a segment of the gene encoding the N. crassa FeS protein as a probe for the corresponding gene of R. sphaeroides. This probe was successfully used to locate the FeS protein gene of R. sphaeroides, which was found to be included in an operon together with the genes for cytochrome b and cytochrome c₁. It is proposed to name this operon fbc, in accordance with the nature of the prosthetic groups FeS, heme b and heme c carried by the encoded subunit polypeptides and also with the order in which the genes occur in the operon. It is further proposed to name the gene for the FeS protein fbcF, for the cytochrome b fbcB and for cytochrome c₁ fbcC.

Results

Selection and analysis of RSF plasmids

The identification of the gene encoding the FeS protein of the b/c₁ complex of R. sphaeroides was achieved by means of cross-hybridization with part of the nuclear gene encoding the corresponding FeS protein of N. crassa (FeS protein probe, see Materials and methods).

The products loaded on lane D were synthesized in the presence of L-[35S]methionine shown in Fig. 2. Fluorogram of the polypeptides synthesized in the R. sphaeroides in vitro system and immunoadsorbed with specific antibodies to each subunit of the R. sphaeroides b/c1 complex. Lanes A and B show the polypeptides synthesized under the direction of pRSF1. The samples loaded on lane A were incubated at 95°C for 3 min and those loaded on lane B were incubated at 20°C for 15 min in loading buffer. Lane C shows the polypeptides whose synthesis was directed by pRSF2. The products loaded on lane D were obtained when 1% SDS was added simultaneously with pRSF1 at the beginning of the 75 min incubation at 37°C. Lane E shows the products obtained with the water blank. The labelled polypeptides of lane B were immunoadsorbed with antibodies specific to the cytochrome b (lane F) and respective control serum (lane G); with antibodies specific to the cytochrome c1 (lane H) and respective control serum (lane I); with antibodies specific to the FeS protein (lane L) and respective control serum (lane M) and with antibodies specific to the 10-kd polypeptide (lane N). The distance of migration of the following marker proteins are indicated on the left hand side of the figure: lysozyme (mol. wt. 14,300), soybean trypsin inhibitor (mol. wt. 21,500), carthionic anhydrase (mol. wt. 30,000), ovalbumin (mol. wt. 46,000). The b/c1 complex preparation from R. sphaeroides GA was run in parallel on the same gel. The polypeptide components indicated by the arrows are: cytochrome b (40 kd), cytochrome c1 (33 kd), FeS protein (24 kd) and the 10-kd polypeptide. Reaction centre polypeptides are present in substoichiometric amount in the b/c1 preparation (Gabellini and Hauska, 1983b).

pBR322 derivatives, bearing partial Sau3A fragments (5-9 kb) of R. sphaeroides genome, were used to transform Escherichia coli. Fourteen positive clones were selected from a total of 10,000 by means of colony filter hybridization. The plasmids (pRSF1-14) isolated from the positive clones were shown, by restriction analysis, to contain a series of overlapping DNA fragments that covered a 13.5-kb region of the R. sphaeroides chromosome. The inserts of pRSF1 and pRSF13 defined the two ends of this region (Fig. 1). A Southern blot of restricted R. sphaeroides genomic DNA, hybridized with all the pRSF plasmids, confirmed the identity and co-linearity of the cloned inserts with directly isolated genomic DNA. A detailed map of restriction sites for SalI, PstI and EcoRI endonucleases was constructed for 10 kb of R. sphaeroides DNA included in the RSF plasmids (Fig. 1). The cross-hybridizing region was more precisely defined by further Southern blot analysis of restriction digests of pRSF8, hybridized with the N. crassa FeS protein probe. Cross-hybridization was obtained with EcoRI 4000-bp, PstI 1100-bp, and SalI 900-bp fragments. The determined DNA sequence showed (70%) homology with the corresponding region of the N. crassa gene chosen as probe. This region encoded an amino acid sequence that was 76% homologous with the related region of the N. crassa FeS protein (Fig. 3).

Fe-S-protein

R. sphaeroides

EVLVVLGVCSCLDVPQDKSKSDDGFQDPDGSHYDGAGHYRGPALNLDP

N. crassa (AA 134 - 183)

Cytochrome b

R. sphaeroides

PTPKINLNNNWLGIVLAFTLVQTVGTILVAMHYTPH

Yeast (AA 20 - 60)

Cytochrome c1

R. sphaeroides

DOAQLRFGDYSEVSTCASHKVFPICTL

Yeast (AA 86 - 115)

In vitro expression of the genes of the RSF plasmids

RSF plasmids were used as templates for a cell free transcription-translation system prepared from R. sphaeroides L. (Chory and Kaplan, 1982) to determine whether the plasmids directed the synthesis of the whole FeS protein gene and also whether a single plasmid directed the synthesis of more than one b/c1 complex subunit. A homologous in vitro system was chosen since it had already been reported that R. sphaeroides DNA is not expressed in E. coli systems (Kaplan et al., 1982). It was confirmed that no expression of cloned R. sphaeroides genes can be obtained using a E. coli in vitro coupled transcription-translation system.

Plasmid RSF1 carrying 9 kb of R. sphaeroides DNA (Fig. 1) was chosen for the experiment of in vitro protein synthesis in the presence of [35S]methionine shown in Figure 2.
under the direction of pRSF1 (lane B) were confirmed by methods of immunoadsorption using antibodies specific for each of the subunits of the R. sphaeroides b/c\(\delta\) complex (see lanes F, H, L). Antibodies raised against the 10-kd polypeptide of the b/c\(\delta\) complex of R. sphaeroides did not react with any polypeptide whose synthesis was directed in vitro by pRSF1 (lane N), suggesting either that the gene encoding the 10-kd polypeptide is not expressed in this system, or (in what seems to be the more likely explanation) that it is not located in the vicinity of the other genes.

The sizes of the cytochrome \(c_1\) and of the FeS protein synthesized in vitro were slightly greater than those of the respective polypeptides of the isolated b/c\(\delta\) complex (Figure 2, lane O), indicating that these two subunits could be synthesized initially as precursors with mol. wts. 1–2 kd greater than those of the mature proteins. All RSF plasmids tested directed the synthesis of the FeS protein, while pRSF8 also directed the synthesis of incomplete cytochrome \(c_1\) (data not shown). The fact that pRSF1, which bears more of the chromosomal DNA indicated on the right hand side of the restriction map (Figure 1) than the other plasmids, directed the full synthesis of all three b/c\(\delta\) complex polypeptides provided a first indication of the organization of the genes.

**Mapping of the fbc genes on pRSF**

Precise information about the organization and primary structure of the fbc genes was obtained by partial DNA sequencing of the RSF plasmids. Comparison of the encoded amino acid sequences with those of the homologous polypeptides from mitochondria and chloroplasts helped with the identification of the correct coding sequences. DNA sequencing of the 900-bp Sall fragment identified by Southern blot analysis using the N. crassa FeS protein probe, revealed the existence of an open reading frame which was highly homologous to the N. crassa FeS protein probe sequence. The amino acid sequence encoded by this region of R. sphaeroides DNA which was homologous to the N. crassa probe showed 76% homology with the N. crassa FeS protein (Figure 3). This allowed initial identification and localization of the R. sphaeroides FeS protein gene (fbcF). Extending the partial DNA sequencing analysis further downstream from the 900-bp Sall fragment led to the identification of the reading frames encoding cytochromes b and \(c_1\). This relative location of these two genes had already been indicated by the in vitro expression data. A TGA stop codon in-frame with the FeS protein sequence was found in the adjacent 250-bp Sall fragment (Figure 1). This stop codon was separated by 12 nucleotides from the initial ATG codon of a new reading frame (Figure 4), that showed high homology with the amino acid sequences of the b cytochromes of mitochondria and chloroplasts. The first stretch of homologous amino acid sequence of this reading frame, comprising the first hydrophobic \(\alpha\)-helix of cytochrome b (Widger et al., 1984; Saraste, 1984), is given in Figure 3, where it is compared with the yeast mitochondrial cytochrome b sequence (Nobrega and Tzagoloff, 1980). The proposed reading frame of the cytochrome \(b\) gene (fbcB) terminated with a TGA stop codon 359 bp downstream of the Sall site in the Sall–Pstl 1300-bp fragment (Figure 1).

It was separated by 17 nucleotides from the initial ATG codon (Figure 4) of a third open reading frame which was shown to be that of the cytochrome \(c_1\) gene (fbcC). Approximately 300 bp downstream from the start codon, the characteristic sequence of a conserved heme binding site of a \(c\) type cytochrome: cys-ser-thr-cys-his (Wakabayashi et al., 1980) was found. There was also high amino acid sequence homology with the yeast cytochrome \(c_1\) in the same heme binding region, as shown in Figure 3 (Sadler et al., 1984).

The intergenic sequences between fbcF and fbc\(\delta\), and between fbc\(\delta\) and fbcC (Figure 4), included Shine-Dalgarno like consensus sequences (Shine and Dalgarno, 1975) that were highly complementary to the sequence of the 3' end of the 16S rRNA of R. sphaeroides.

**Identification of a 2.9 kb mRNA bearing all three fbc cistrons**

The in vitro expression of RSF plasmids, taken together
with the DNA sequencing data, indicated that the genes fbcF, fbcB and fbcC, lie in a single operon. Confirmation of this was sought by performing Northern blot analysis with total R. sphaeroides RNA. The identification of a single mRNA species was achieved by hybridization with different segments of a 5-kb DNA region including the fbc genes (Figure 5). The 5-kb region was subcloned as five PstI fragments in pBR322. The fragments were numbered 1-5 according to their order in the PstI restriction map as shown in the lower part of Figure 5. The nick-translated plasmids were used individually as hybridization probes. The evaluation of the size of the mRNA hybridizing with the fbc genes was estimated by comparing its migration rate against the rates of single-stranded Tagl and Hincl II DNA fragments derived from pBR322 (Figure 5, lanes A, B). Hybridization was clearly obtained between a 2.9-kb mRNA species and the PstI fragment 2 of 1100 bp (Figures 5, lane 2). This fragment comprises all of fbc (-600 bp), together with the first 170 bp of fbcB. Stronger hybridization was observed between the same mRNA species and the PstI fragment 3 of 2300 bp (Figure 5, lane 3). Fragment 3 comprises the following 1100 bp of fbcB and approximately all of fbcC.

Only one mRNA species of 2.9 kb was also obtained by hybridization with the whole pRSF1 and pRSF8 (not shown). The smear in the 2.5-kb region, particularly evident in lane 3 of Figure 3, most likely reflects some partial degradation of mRNA during the isolation procedure.

The PstI fragments 1, 4 and 5 flanking the fbc genes did not engage in detectable hybridization with any mRNA species. The shadow observed in the region of 1.3 kb, best visible in lane 1, represents some unspecific binding to the large rRNA start codon was unsuccessful. The apparent synthesis of precursor forms of the FeS protein and cytochrome c_in vitro could indicate that these two polypeptides may undergo processing before being integrated into the membrane. This might be expected from the location of the functional domains of the FeS protein and cytochrome c_in the P side (Hauska et al., 1983) of the membrane, corresponding in R. sphaeroides to the outer surface of the cytoplasmic membrane. Thus the precursors may contain signal sequences responsible for directing transport across the cytoplasmic membrane.

The present paper shows that three subunits present in the b/c1 complex preparation from R. sphaeroides are encoded by an operon. This observation leads to the question whether this operon alone encodes the functional ubiquinol-cytochrome c oxidoreductase and thus whether the 10-kd polypeptide present in the b/c1 preparation plays, for example, only a regulatory role or is even a contaminant. In E. coli most of the defined multisubunit enzymes are fully encoded by operons such as the atp (Futai and Kanazawa, 1983; Walker et al., 1984), ace (Guest and Stephens, 1980) and sdh (Wood et al., 1984) operons. In photosynthetic bacteria, by contrast, the genes for the F4 and F1 portions of the ATP synthase are encoded in separate regions of the chromosome (Tetyukiewicz et al., 1984). Moreover, the organization of the genes of the photosynthetic reaction center and light harvesting complex 1 of R. capsulata (Youvan et al., 1984) and R. sphaeroides (Williams et al., 1983), does not correspond to the polypeptide composition of the isolated enzyme complexes.

Thus the observation that the three R. sphaeroides fbc genes are organized in an operon does not constitute a sufficient basis to conclude that the functional unit of the b/c1 complex comprises only three polypeptides. Rather, one can only draw analogy to the structure of the functional chloroplast b6/f complex (Hurt and Hauska, 1982), which is effectively equivalent to a three subunit structure of the R. sphaeroides complex.

Materials and methods
Cloning of R. sphaeroides genes and selection of RSF plasmids
R. sphaeroides GA genome DNA was isolated essentially as described by Barker (1982) and subjected to partial digestion by Sau3A. Restriction fragments of size 5-9 kb were isolated by 0.8% agarose gel electrophoresis and ligated into the 5' dephosphorylated BamHI site of pBR322. The plasmids so obtained were used to transform E. coli SK (Hananah, 1983). The selection of RSF plasmids was performed by colony filter hybridization (Grinstein and Hogness, 1975). The DNA probe used for hybridization was isolated from a cDNA clone of N. crassa FeS protein mRNA. The probe (FeS protein probe) was a 149-bp RNA-Xhol fragment end-labelled cDNA fragment, encoding a cluster of four cysteines which are most probably involved in forming the FeS center (Figure 3; Harnisch et al., in preparation). The probe was 5' end-labelled with [γ-32P]ATP (c>5000 Ci/mmol, Amersham) and used at a concentration of 3-4 Ci/20 ml
hybridization buffer. The hybridization conditions were kept at low stringency by incubation in 5 x NaCl-citrate (SSC), 0.1% SDS, 0.2% bovine serum albumin, Ficoll, polyvinylpyrrolidone, 50% formamide (reagent grade Merck) for 42 h at 20°C. After incubation the filters were washed several times in 5 x SSC at progressively increasing temperatures up to 45°C. Autoradiography was performed with Agfa-Gevaert X-ray film. Plasmid DNA was isolated (Maniatis et al., 1982) from E. coli overnight cultures grown at 37°C in LB medium plus Ampicillin 50 μg/ml. Restriction enzymes were used according to manufacturers’ specifications.

Southern blotting analysis was performed by standard techniques (Maniatis et al., 1982) except that the condition of hybridization with the heterologous probe was kept at low stringency as described for colony filter hybridization.

*In vitro transcription and translation system and immunodetection*

The *in vitro* expression of *R. sphaeroides* cloned genes was tested in a S30 extract prepared from *R. sphaeroides* L strain that lacks the *Kfr* restriction system (Chory and Kaplan, 1982; Zubay, 1973; Prat, 1980). Cells from an exponentially growing photosynthetic culture were broken by passage through a Sorvall Ribi Cell Fractionator at 12 000 p.s.i. and 4°C. *In vitro* protein synthesis was carried out in the presence of 19 unlabelled amino acid (0.22 μM each) plus [35S]-methionine (Amersham); 50 μCi in a 50 μl reaction mixture. Aliquots of 5 μl of the assay mixture were loaded onto 12% SDS-polyacrylamide gels (Laemmli, 1970). Rabbit antisera were raised against each of the four SDS-denatured subunits of the *R. sphaeroides* b/c complex. The subunits synthesized in vitro were immunorecognizable from 40 μl of solubilized *R. sphaeroides* in vitro assay (Goldman and Blobel, 1978), using 5 μg of protein A-Sepharose CL-4B (Pharmacia) and 10 μl of antisera. Incubation of the samples at high temperature, causing irreversible aggregation of cytochrome b, was avoided. The 35S-labelled polypeptides were visualized by fluorography (Bonner and Laskey, 1974) using Kodak X-Omat AR film.

**References**


The *fbc* operon encoding subunits of the b/c complex from *R. sphaeroides*


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