ELSEVIER

Biochimica et Biophysica Acta 1276 (1996) 246–252

BBA Biochimica et Biophysica Acta

Some characteristics of cytochrome *f* in the cyanobacterium *Phormidium laminosum*: its sequence and charge properties in the reaction with plastocyanin

M.J. Wagner, J.C.L. Packer, C.J. Howe, D.S. Bendall *

Department of Biochemistry and Cambridge Centre for Molecular Recognition, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, UK

Received 20 February 1996; revised 19 June 1996; accepted 26 June 1996

Abstract

Part of the *pet*CA operon was cloned and the sequence of the cytochrome *f* gene from the moderately thermophilic cyanobacterium *Phormidium laminosum* determined. A partial sequence of the *pet*C gene encoding the Rieske iron-sulphur protein was also obtained. The cytochrome *f* gene encodes a mature protein of 385 residues and a leader sequence of 45 residues. The mature protein contains several acidic or neutral residues corresponding to basic residues in the turnip protein. Some of the latter are thought to be important for the interaction with plastocyanin via its 'eastern' face. Many of the corresponding residues on the eastern face of *P. laminosum* plastocyanin are either basic or neutral instead of acidic. These comparisons suggested that the local charges on *P. laminosum* cytochrome *f* that are important for its interaction with the homologous plastocyanin may be negative rather than positive. The importance of acidic groups was confirmed by measuring the rates of reduction of horse heart cytochrome *c* and *P. laminosum* and spinach plastocyanins by the cytochrome *bf* complex isolated from *P. laminosum*. *P. laminosum* plastocyanin gave the highest rates, which decreased at high ionic strength, confirming the importance of positive local charges on this protein. When extrapolated to infinite ionic strength the rates observed with the two kinds of plastocyanin were similar, but cytochrome *c* became unreactive. An optimum was observed in the ionic strength response with *P. laminosum* plastocyanin.

Keywords: Cytochrome *f*; Rieske protein; Cytochrome *bf* complex; Plastocyanin; Cyanobacterium; Electron transfer

1. Introduction

Cytochrome *f* and plastocyanin are part of the electron transfer chain between Photosystems II and I. Cytochrome *f* is the largest of the four polypeptides of the cytochrome *bf* complex, which mediates electron transfer between plastoquinol and plastocyanin or cytochrome *c*-553 [1]. The other components are cytochrome *b*-563, the [2Fe-2S] Rieske iron-sulphur protein and subunit IV. The genes encoding the proteins of the complex are organised in two operons in cyanobacteria [2], the *pet*CA operon, which encodes cytochrome *f* and the Rieske protein and the *pet*BD operon encoding cytochrome *b*-563 and subunit IV.

Several sequences of the cyanobacterial Rieske iron-sulphur protein are known [2–4]. The protein is divided into two domains with different functions. The N-terminal domain contains a hydrophobic stretch, which is responsible for the association of the Rieske protein with the membrane and the rest of the cytochrome *bf* complex [5–8]; the C-terminal domain contains the ligands for the iron-sulphur cluster. Sequence alignments between the Rieske protein from the cytochrome *bf* complex and Rieske proteins from the analogous cytochrome bc_1 complex have revealed two completely conserved regions, a hexa- (CPCHGS) and a heptapeptide (CTHLGCV) which contain the four ligands to the iron atoms in the [2Fe-2S] cluster [9].

The second protein encoded by the *pet*CA operon is cytochrome *f*. The crystal structure of the lumenal part of turnip cytochrome *f* shows that the protein is a long, thin, flat molecule, composed of two domains, the larger being adjacent to the membrane-spanning polypeptide in the holoprotein [10,11]. This large domain also contains the haem. The high degree of sequence conservation in cytochrome *f* from different sources [12], including cyanobacteria, suggests that the folding pattern of

* Corresponding author. Fax: +44 1223 333345; e-mail: dsb4@bioc.cam.ac.uk.

PII S0005-2728(96)00084-9

cyanobacterial cytochrome *f* is essentially the same as that of the turnip protein.

Plastocyanin, a blue copper protein, is located in the thylakoid lumen and acts as a soluble electron carrier between cytochrome *f* and $P700^+$. The tertiary structures of a number of higher plant and algal proteins are known and they are found to be conserved [13–18]. The protein is an eight-stranded, anti-parallel β-barrel with the shape of a slightly flattened cylinder roughly of the dimensions $40 \times 32 \times 28$ Å. It contains a single type 1 copper atom. Most higher plant proteins feature two acidic patches on the 'eastern' face of the molecule. The second distinct feature of the protein is the so-called hydrophobic patch on the 'northern' face of the molecule. Two possible routes of electron transfer from cytochrome *f* to the copper in plastocyanin have been identified in the past [19,20]. One possible route involves the surface exposed copper ligand His-87 at the 'northern' end of the molecule. The second route involves the solvent exposed, highly conserved Tyr-83. While the electron transfer route via His-87 should be favoured by the shorter distance between the surface and the copper atom, there has been strong experimental evidence by site-directed mutagenesis of the highly conserved Tyr-83 [21,22] that this route is in fact the favoured one. Sequence similarity strongly suggests that cyanobacterial plastocyanins will be found to have an overall structure similar to that of their plant and algal counterparts. Recently the structure of *Anabaena variabilis* plastocyanin (Freeman, personal communication, [23]) has been reported and shows a remarkable similarity in its over-all fold to the algal and higher plant proteins. However, the cyanobacterial proteins are much less acidic, and only vestigial acidic patches are evident on the 'eastern' face.

A number of basic residues in turnip cytochrome *f* form a positive ridge between the small and large domains [24]. Cramer et al. have suggested that electrostatic interaction between this positive patch and the negative patches of plastocyanin are important in the electron transfer reaction. The aim of the current work was to study how this model might apply to *Phormidium laminosum* in view of the known charge characteristics of its plastocyanin [25]. The gene for cytochrome *f* was cloned and sequenced so that the deduced amino acid sequence could be compared with that of plastocyanin by making use of the higher plant structures and models. The electrostatic characteristics of the interaction were then studied by measuring rates of electron transfer as a function of the ionic strength in vitro.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Phormidium laminosum strain OH-1-p clone 1 was grown in medium D [26], supplemented with $NaHCO_3$ (0.1 g/l). For isolation of plastocyanin the medium was also supplemented with 0.1 mg/l $CuSO_4$. Cells were grown in an orbital incubator at 45°C under continuous illumination (by five 30 Watt fluorescent tubes) in an atmosphere enriched with 5% CO_2. The *E. coli* strain used for cloning was TG1 [27].

2.2. Isolation and purification of proteins

The procedure of Varley et al. [25] was used for the isolation of *P. laminosum* plastocyanin. Spinach plastocyanin was isolated as previously described [28]. *P. laminosum* cytochrome *f* was isolated from thylakoid fragments prepared as described earlier [29] by ethyl acetate extraction [30]. The final acetone precipitate was dissolved in 60 mM Na_2HPO_4 as described by Davenport and Hill [31]. The crude extract was subjected to SDS PAGE to separate cytochrome *f* from the other proteins for sequence analysis. C-type cytochromes were identified using the method of Thomas et al. [32] and cytochrome *f* was identified as a haem containing protein of approx. 33 kDa molecular mass. The proteins were blotted onto a PVDF membrane. Protein sequencing was carried out by the Protein Sequencing Facility of the Department of Biochemistry, University of Cambridge.

P. laminosum cytochrome *bf* complex was prepared following the procedure given by Hurt and Hauska [1] with slight modifications using MEGA-9 as detergent for solubilization of the thylakoids.

2.3. PCR amplification of cytochrome f gene fragment

A forward PCR primer was designed from the N-terminal amino acid sequence obtained for the cytochrome *f* protein from *P. laminosum* (Fig. 1). The reverse primer was designed from a highly conserved region (see Fig. 1) in cytochrome *f* protein sequences. *Hin*dIII restriction sites were incorporated at the ends of both primers in order to facilitate cloning of the generated PCR products. Degeneracy in the primers was reduced by taking into account the codon bias for the genes encoding plastocyanin [25] and the 9 kDa extrinsic polypeptide for photosystem II from *P. laminosum* [33]. The sequence of the forward primer was 5′-AAAAAGCTTACCCGTTTTGGCCCAACAAAATTAC-3′; the sequence of the reverse primer was 5′-AAAAAGCTTCTGCCAAAGCCGCCCACATTCGGATTATTGGT-3′. Genomic DNA prepared as described elsewhere [25] was used as template. Forty cycles in the presence of 2.5 mM $MgCl_2$ were used; the program used a denaturing temperature of 92°C for 1 min, an annealing temperature of 55°C for 1 min and an extension at 72°C for 1 min. PCR products were purified on agarose gels [34]. They were then cut with *Hin*dIII, cloned into M13mp18 RF DNA and sequenced. Single-stranded M13mp18 DNA containing the cytochrome *f* gene fragment was subjected to amplification by PCR (using an annealing temperature of 60°C). The PCR product was then used for random prime labelling and subsequently as a probe with ECL.

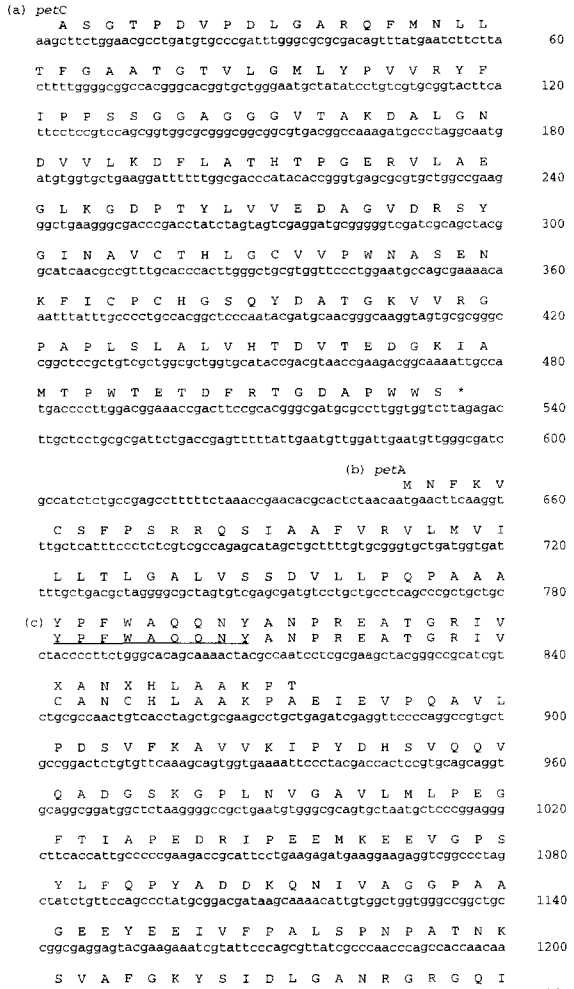

Fig. 1. Nucleotide sequence of part of the *P. laminosum pet* CA operon. The sequence is aligned with (a) the deduced amino acid sequence of part of *pet*C, (b) the deduced amino acid sequence of *pet*C and (c) the partial amino acid sequence obtained from the mature protein. X represents a residue which was not identified from protein sequencing. The regions of the protein sequence to which PCR primers were made are underlined.

2.4. DNA manipulations

P. laminosum genomic DNA was prepared from cells grown to mid-logarithmic phase [33]. The method was modified in that the final CsCl gradient centrifugation was omitted and genomic DNA precipitated. Digestion of genomic DNA with restriction enzymes, gel electrophoresis, cloning into pUC and M13 vectors and bacterial transformation were carried out as described by Sambrook et al. [34].

2.5. Library construction and screening

About 30 μg genomic DNA was digested with *Hin*dIII (Boehringer), and fragments in the size range of 2-4 kb were extracted following electrophoresis essentially as described by Howe et al. [35]. Purified genomic DNA fragments were ligated into pUC18 cut with *Hin*dIII and dephosphorylated with shrimp alkaline phosphatase (USB). Ligated DNA was transformed into competent *E. coli* TG1, and plated onto medium containing ampicillin, IPTG and X-Gal [34]. About 90% of the transformants were recombinant.

Colony lifts onto nitro-cellulose filters (Hybond ECL, Amersham International) were prepared and probed with the amplified gene fragment using the Amersham ECL system, according to the manufacturer's instructions. Positive colonies were picked and plasmid DNA prepared [34]. The insert DNA was then subcloned into M13mp18.

2.6. DNA sequencing

DNA sequencing was performed using single-stranded M13 DNA and a Sequenase kit (USB) according to the manufacturer's instructions. DNA and derived amino acid sequences were analysed using the STADEN and Genetics Computer Group (GCG) packages [36].

2.7. Determination of oxidation reduction potentials

The determination of the oxidation reduction potential of *P. laminosum* plastocyanin was performed using a cuvette for redox potentiometric titration as described by Dutton [37]. The reference electrode was a combination calomel electrode (Russell). The titration was performed under an argon atmosphere to exclude oxygen. The potential of the calomel electrode given by Dutton [37] was assumed and the titration carried out at 296°K.

The buffer for the titration was 50 mM KCl 50 mM MOPS (pH 7.0). Spectra were recorded between 570 and 730 nm throughout the titration with a single beam spectrophotometer to assess the redox state of the protein. The protein sample was fully oxidised with 40 mM ferricyanide at the beginning of the titration. The potential was then adjusted to lower potentials by addition of aliquots of freshly prepared dithionite solution. To correct for drifts in the baseline of the spectrophotometer, the difference in absorbance at 598 and 711 nm was calculated.

2.8. Assay of plastoquinol:plastocyanin oxidoreductase activity

The initial rates of reduction of spinach and *P. laminosum* plastocyanins and horse heart cytochrome *c* (Sigma C-2506) were assayed in a Cary 219 spectrophotometer at 598 nm and 550 nm respectively. The assay buffer contained 0.1% (w/v) digitonin, 20 mM MES pH 6.2, 4 nM *P. laminosum* cytochrome *bf* complex and 8 μM plastocyanin or cytochrome *c*. The experiments were carried out at room temperature, the assay initiated by the addition of 200 μM plastoquinol-1 for cytochrome *c* and 40 μM plastoquinol-1 for plastocyanin and the initial rate of reduction determined. Plastoquinol-1 was synthesised following the procedure given by Wood and Bendall [38]. Extinction coefficients of 18500 ($\Delta \epsilon_{red-ox}$.) $M^{-1}cm^{-1}$ at 550 nm [39] for cytochrome *c* and 4700 $M^{-1}cm^{-1}$ for plastocyanin at 598 nm [22] were used for the determination of the protein concentrations. The concentration of the cytochrome *bf* complex was assayed by the absorbance of cytochrome *f* at 556 nm for *P. laminosum* protein [40].

A 25-fold excess of plastoquinol-1 over the cytochrome *c* and an eight-fold excess of plastoquinol-1 over the plastocyanin concentration were used for the measurements, therefore keeping cytochrome *f* in the *bf* complex reduced. Measurements with concentrations of the redox proteins varying from 2 to 20 μM (data not shown) showed a linear dependence of the initial rate of reduction (v_{in}) on the redox-protein concentration in the assay. The ionic strength was adjusted by addition of 5 M NaCl to the reaction buffer, leading to final concentrations of NaCl in the reaction mixture ranging from 0–1 M NaCl.

The rates were corrected by subtraction of the uncatalysed rates of reduction of plastocyanin and cytochrome *c* by plastoquinol-1.

3. Results

3.1. Purification and N-terminal sequencing of the cytochrome f protein

Cytochrome *f* was isolated from mid-log-phase cultures of *P. laminosum* grown in medium D and subjected to SDS PAGE. The identity of cytochrome *f* was determined by its molecular weight on a gel stained for haem [32]. The N-terminal sequence was determined and is shown in Fig. 1.

3.2. Gene cloning

PCR from genomic DNA generated a product of the predicted size of approx. 700 base pairs, which was cloned into M13. Sequencing confirmed that the fragment contained a partial *pet*A gene sequence. The fragment was re-amplified by PCR and used to probe a Southern blot of genomic DNA digested with various restriction enzymes. This indicated a *Hin*dIII fragment of 2–3 kbp on a Southern blot of the genomic digest. Fragments of corresponding size were isolated and cloned into pUC18 and used to transform *E. coli* TG1. Screening of a genomic library with the PCR fragment identified clones containing plasmids with 2500 bp inserts. Restriction mapping, subcloning and partial sequencing confirmed that the *pet*A gene was entirely contained within the plasmid. Sequencing of the fragment also yielded part of the *pet*C sequence.

3.3. The petCA operon

The nucleotide sequence for the part of the *P. laminosum pet*CA operon cloned is shown in Fig. 1. The first 534 bp of the cloned fragment encode the C-terminal 177 amino acids of the Rieske protein. The first residues of the Rieske protein are not encoded within the cloned fragment. The protein sequence obtained from the cloned DNA fragment is aligned with the cyanobacterial sequences in Fig. 2. The level of conservation for the protein is 59% amongst the cyanobacterial sequences with the C-terminal half being more highly conserved than the N-terminal half. This is consistent with the function of the C-terminus in housing the iron sulphur cluster of the protein. The hexa-(CPCHGS) and heptapeptide

```
                10         20         30         40         50         60
phola  ---------- -----ASGTP PDVDLGARQF MNLLTFGAAT ATVLGMLYPV VRYFIPPSSG
syny3  MLVKILKFRR PIMTQISGSP DVPDLGRRQF MNLLTFGTIT GVAAGALYPA VKYLIPPSSG
synp2  ---------- --MTQLSGSS DVPDLGRRQF LNLLWVGTAA GTALGGLYPV IKYFIPPSSG
nossp  ---------- --MAQFSESA DVPDMGRRQF MNLLTFGTVT GVALGALYPV VKYFIPPASG
                           *    ***    ***  ***  *        * ***    * *** **

                70         80         90         100        110        120
phola  GAGGGVTAKD ALGNDVVLKD FLATHTPGER VLAEGLKGDP TYLVVEDAGV DRSYGINAVC
syny3  GSGGGVTAKD ALGNDVKVTE FLASHNAGDR VLAQGLKGDP TYIVVQGDDT IANYGINAVC
synp2  GAGGGVIAKD ALGNDIIVSD YLQTHTAGDR SLAQGLKGDP TYVVVEGDNT ISSYGINAIC
nossp  GAGGGTTAKD ELGNDVSLSK FLENRNAGDR ALVQGLKGDP TYIVVENKQA IKDYGINAIC
       * ***  ***  ****       *     * *  *  ****** ** **         ***** *

                130        140        150        160        170        180
phola  THLGCVVPWN ASENKFICPC HGSQYDATGK VVRGPAPLSL ALVHTDVTED GKIAMTPWTE
syny3  THLGCVVPWN ASENKFMCPC HGSQYNAEGK VVRGPAPLSL ALAHATVTDD DKLVLSTWTE
synp2  THLGCVVPWN TAENKFMCPC HGSQYDETGK VVRGPAPLSL ALVHAEVTED DKISFTDWTE
nossp  THLGCVVPWN VAENKFKCPC HGSQYDETGK VVRGPAPLSL ALAHAN-TVD DKIILSPWTE
       **********   **** *** *****   ** ********** ** *   * *  *     ***

                190
phola  TDFRTGDAPW WS
syny3  TDFRTDEDPW WA
synp2  TDFRTDEAPW WA
nossp  TDFRTGDAPW WA
       *****   ** *
```

Fig. 2. Sequence alignment of cyanobacterial Rieske protein sequences. The sequences used are *P. laminosum* (phola), *Synechocystis* sp. PCC6803 (syny3), *Synecococcus* sp. PCC7002 (synp2) and *Nostoc* sp. (nossp). Conserved residues are marked with an asterisk (*). Dashes indicate gaps introduced to improve the alignment. The sequences were aligned using the 'clustal V' multiple alignment program. All the sequences except that of the *P. laminosum* one were extracted from the 'Swissprot' database.

```
          <------------leader sequence---------------->
phola     MNFKVCSFPSRRQSIAAFVRVLMVILLTLGALVSSDVLLPQPAAAYPFWAQQNY-ANPRE
nossp     MR-NASVTARLTRSVRAIVKTLLIAIATVTFYFSCDLALPQSAAAYPFWAQQTYPETPRE
synp2     MK-TPELMAIWQR----LKTACLVAIATFGLFFASDVLFPQAAAAYPFWAQQTAPETPRE
syny3     MR-NPDTLGLWTKTMVALRRFTVLAIATVSVFLITDLGLPQAASAYPFWAQETAPLTPRE
          *                          *     *   ** * *******      ***

phola     ATGRIVCANCHLAAKPAEIEVPQAVLPDSVFKAVVKIPYDHSVQQVQADGSKGPLNVGAV
nossp     PTGRIVCANCHLAAKPTEVEVPQSVLPDTVFKAVVKIPYDTSAQQVGADGSKVGLNVGAV
synp2     ATGRIVCANCHLAAKEAEVEIPQSVLPDQVFEAVVKIPYDHSQQQVLGDGSKGGLNVGAV
syny3     ATGRIVCANCHLAQKAAEVEIPQAVLPDTVFEAVVKIPYDLDSQQVLGDGSKGGLNVGAV
           ************ *  * * ** **** ** ********   ***  ****  ******

phola     LMLPEGFTIAPEDRIPEEMKEEVGPSYLFQPVADDKQNIVAGGPAAGEEYEEIVFPALSP
nossp     LMLPEGFKIAPEDRISEELQEEIGDTY-FQPYSEDKENIVIVGPLPGEQYQEIVFPVLSP
synp2     LMLPDGFKIAPADRLSDELKEKTEGLY-FQSYAPDQENVVIIGPISGDQYEEIVFPVLSP
syny3     LMLPEGFKIAPPDRLSEGLKEKVGGTY-FQPYREDMENVVIVGPLPGEQYQEIVFPVLSP
          **** ** *** **       *     * ** *  *  * *  **  *  * ***** ***

phola     NPATNKSVAFGKYSIDLGANRGRGQIYPTGEKSNNAVYNASAAGVITAIAKAEDGSARVK
nossp     NPATDKNIHFGKYSVHVGGNRGRGQVYPTGEKSNNNLYNASATGTIAKIAKEEDEDGNVK
synp2     DPKTDKNINYGKYAVHLGANRGRGQVYPTGELSNNNQFKASATGTITNIAVNEAAG----
syny3     DPAKDKSINYGKFAVHLGANRGRGQIYPTGLLSNNNAFKAPNAGTISEVNALEAGG----
           *   *    **      * ****** ****  ***     *    * *      *

phola     ----IRTEDGTTIVDKIPAGPELIVSEGEEVAAGAALTNNPNVGGFGQKDTEIVLQSPNR
nossp     YQVNIQPESGDVVVDTVPAGPELIVSEGQAVKAGDALTNNPNVGGFGQRDAEIVLQDAGR
synp2     TDITISTEAGEVIDT-IPAGPEVIVSEGQAIAAGEALTNNPNVGGFGQKDTEVVLQNPAR
syny3     YQLILTTADGTEIVD-IPAGPELIVSAGQTVEAGEFLTNNPNVGGFGQKDTEVVLQNPTR
                  *       ***** *** *    **  ************ * * ***   *

phola     VKGRIAFLAAITLTQILLVLKKKQVERVQAAEM-TF
nossp     VKGLIAFVALVMLAQVMLVLKKKQVERVQAAEM-NF
synp2     IYGYMAFVAGIMLTQIFLVLKKKQVERVQAAGNCDF
syny3     IKFLVLFLAGIMLSQILLVLKKKQIEKVQAAEL-NF
             * *   * *  ******* * ****    *
```

Fig. 3. Sequence alignment of cyanobacterial cytochrome *f* sequences. The sequences used are *P. laminosum* (phola), *Synechocystis* sp. PCC6803 (syny3), *Synecococcus* sp. PCC7002 (synp2) and *Nostoc* sp. (nossp). Identical residues are marked with an asterisk (*). Dashes indicate gaps introduced to improve the alignment. The sequences were aligned using the 'clustal V' multiple alignment program. All the sequences except the *P. laminosum* one were extracted from the 'Swissprot' database.

(CTHLGCV) that are conserved throughout the Rieske proteins from bc_1 and *bf* complexes are also conserved within the *P. laminosum* Rieske sequence.

The region encoding mature cytochrome *f* begins at position 782, as shown by comparison with the N-terminal amino acid sequence of the purified protein (Fig. 1). The predicted and determined sequences agree completely. Alignment with other cyanobacterial *pet*A sequences (Fig. 3) suggests that the ATG codon at position 647 is used for translation initiation, although it is preceded by only a rather weak putative ribosome binding site (5′-GAA-3′) complementary to the 3′ end of cyanobacterial 16S rRNAs [41]. This implies that the protein is synthesised as a 35.2 kDa precursor, with a 4.8 kDa leader sequence of 45 residues. As with other cyanobacterial cytochrome *f* sequences, the leader sequence is some 10 residues longer than those in higher plants [2–4]. The cleavage site can be identified unambiguously, with the mature protein comprising 385 residues. Although the level of amino acid sequence conservation among the higher plant proteins is as high as 80% identity [12], the four cyanobacterial sequences show only 44% identity.

3.4. Ionic strength dependence of reduction of redox proteins by the P. laminosum cytochrome bf complex

The effect of change in ionic strength on the initial velocities of reduction of spinach plastocyanin, *P. laminosum* plastocyanin and horse-heart cytochrome *c* by the *P. laminosum* cytochrome *bf* complex are shown in Fig. 4.

At low ionic strength cytochrome *c* was a good acceptor, but the rate declined sharply above $I = 70$ mM. Conversely, spinach plastocyanin was a poor acceptor at low ionic strength but the rate increased as the ionic strength was raised, although the maximum rate observed was not as high as can be achieved with cytochrome *c*. Since the reactive sites on horse heart cytochrome *c* and higher plant plastocyanin are strongly basic and acidic respectively, these results show that the significant local charges on *P. laminosum* cytochrome *f* are negative. *P. laminosum* plastocyanin gave high rates compared with the artificial acceptors, with a maximum at $I = 20$ mM and declining at higher ionic strength. From this one may conclude that complementary charges dominate the interaction between the homologous proteins at $I > 20$ mM and that the significant local charges on *P. laminosum* plastocyanin are positive. Possible explanations for the existence of an optimum in the ionic strength curve are discussed below. At high values of I, when the influence of the charges on the proteins was effectively eliminated, the two plastocyanins gave very similar rates of reaction, whereas cytochrome *c* became almost completely unreactive. The interaction between cytochrome *c* and *P. laminosum* cytochrome *f* therefore depends entirely on long-range electrostatic forces, but both plastocyanins can interact with *P. laminosum* cytochrome *f* in the cytochrome *bf* complex via short-range forces such as hydrogen bonds or hydrophobic interactions.

3.5. Midpoint potential of P. laminosum plastocyanin

To aid the interpretation of the electron transfer measurements described above, the midpoint potential of *P.*

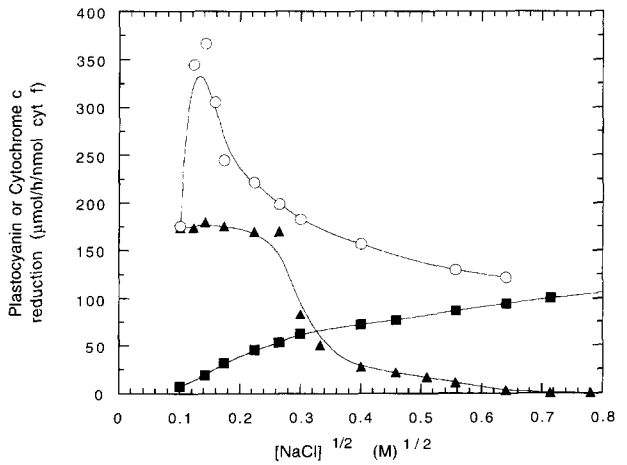

Fig. 4. The effect of ionic strength on the initial rate of reduction of horse heart cytochrome *c* (triangles), *P. laminosum* (open circles) and spinach plastocyanin (squares) by the *P. laminosum* cytochrome *bf* complex. Measurements were carried out at room temperature in 20 mM MES buffer (pH 6.2). The ionic strength was adjusted by addition of 5 M NaCl.

```
phola   YPFWAQQNYA NPREATGRIV CANCHLAAKP AEIEVPQAVL PDSVFKAVVK IPYDHSVQQV   60
brara   YPIFAQQNYE NPREATGRIV CANCHLASKP VDIEVPQAVL PDTVFEAVVK IPYDMQLKQV   60
        **  *****  ********** ******* **   ******** ***** **** ****    **

phola   QADGSKGPLN VGAVLMLPEG FTIAPEDRIP EEMKEEVGPS YLFQPYADDK QNIVAGGPAA  120
brara   LANGKKGALN VGAVLILPEG FELAPPDRIS PEMKEKIG-N LSFQNYRPNK KNILVIGPVP  119
         * * ** ** ***** **** *  ** ***   **** *     ** *   *  **   **

phola   GEEYEEIVFP ALSPNPATNK SVAFGKYSID LGANRGRGQI YPTGEKSNNA VYNASAAGVI  180
brara   GQKYSEITFP ILAPDPATNK DVHFLKYPIY VGGNRGRGQI YPDGSKSNNT VYNATAGGII  179
        *  * ** **  * * *****  * * ** *   * ******* ** * ****  **** * * *

phola   TAIAKAEDGS ARVKI-RTED GTTIVDKIPA GPELIVSEGE EVAAGAALTN NPNVGGPGQK  239
brara   SKILRKEKGG YEITIVDASN ERQVIDIIPR GLELLVSEGE SIKLDQPLTS NPNVGGFGQG  239
          *   * *     *           * **  * ** *****          *  *********

phola   DTEIVLQSPN R  250
brara   DAEIVLQDPL R  250
        * ***** *  *
```

Fig. 5. Sequence alignment between *P. laminosum* (phola) and turnip (brara) cytochrome *f*. Only the sequence of the water soluble crystallised domain of turnip cytochrome *f* was used for the alignment to the *P. laminosum* protein. Identical residues are marked with an asterisk (*). Dashes indicate gaps introduced to improve the alignment. Residues that are positively charged in the turnip sequence but neutral or acidic in the *P. laminosum* sequence are shown bold. Numbering is according to the turnip sequence and the numbers for each sequence are noted at the end of each line. The sequences were aligned using the 'clustal V' multiple alignment program. Fig. 1 Nucleotide sequence of part of the *P. laminosum petCA*.

laminosum plastocyanin was measured and found to be 380 mV under the conditions specified earlier. This value falls within the range (345–395 mV) usually observed for this protein [42].

4. Discussion

The sequence information for *P. laminosum* cytochrome *f* complements that which we have previously reported for plastocyanin from the same organism [25]. Comparison of the two sequences has facilitated the interpretation of the electron transfer reaction between these two proteins described above. This is one of the few studies of electron transfer between cytochrome *f* and plastocyanin in a homologous system.

Fig. 5 shows an alignment between the sequence that forms the lumenal domain of turnip cytochrome *f* which was used for crystallisation and the corresponding sequence from *P. laminosum*. It shows that 63% of the amino acids are identical between the two proteins. Cramer et al. [24] speculated that residues Lys-187, Arg-209, Lys-65, Lys-66 and Lys-58, that form a positive ridge on cytochrome *f*, are involved in plastocyanin binding. They suggested that higher plant plastocyanin would either bind with its acidic patch to these positively charged residues, or at least be directed to that positive site on cytochrome *f*. Electron transfer in this initial complex of the proteins seems to be unlikely, since the distance between the copper and the haem would be too great.

Most of the corresponding residues in the *P. laminosum* sequence are either acidic or neutral; that is Asp-188, Ala-208, Ser-65 and Gln-58. Only Lys-66 is conserved. The aspartate at position 188 is most notable because with higher plant proteins Lys-187 can be cross-linked to Asp-44 of plastocyanin by treatment with a carbodiimide [43]. The equivalent residue in the *P. laminosum* plastocyanin sequence is Lys-46 [25].

Apart from the residues discussed above, there are some other acidic or neutral residues in *P. laminosum* that correspond to basic residues in the turnip protein. These are also highlighted in Fig. 5. The sequence information discussed above together with the kinetic data presented in this paper support the Cramer hypothesis that the residues which form the positive ridge in the turnip protein are involved in the binding of plastocyanin since most of the relevant basic residues are either acidic or neutral in the *P. laminosum* protein.

The information also indicates that the fundamental characteristics of the interaction between *P. laminosum* plastocyanin and cytochrome *f* are likely to be similar, except that the charge characteristics of the two proteins are reversed when compared to the higher plant equivalent.

The above discussion assumes that the lumenal parts of *P. laminosum f* and turnip cytochrome *f* have an almost identical structure. The assumption that acidic residues on *P. laminosum* cytochrome *f* play an important role in the interaction with other redox proteins is backed by the results of the kinetic experiments.

The fact that *P. laminosum* plastocyanin shows an optimum in the ionic strength dependence of the reduction by the *P. laminosum* cytochrome *bf* complex suggests that the interaction and electron transfer between the two proteins is not a simple single step reaction. The electrostatic complex between the two *P. laminosum* proteins at low ionic strength seems to be somewhat less active in electron transfer. The same effect has been observed for the interaction of higher plant plastocyanin with cytochrome *f* [44]. The effect was interpreted as indicating that the electrostatic complex between the two proteins formed at low ionic strength is not suitable for electron transfer. A rearrangement of the two proteins would have to occur prior to the electron transfer reaction. The strong attractive forces between the proteins at low ionic strength would work against a rearrangement of the two proteins, thus slowing down the reaction.

This interpretation is supported by the finding that the product formed by cross-linking between acidic groups on plastocyanin and basic groups on cytochrome *f* in higher plant proteins is inactive in electron transfer [45]. Moreover, the results are consistent with the crystal structure of turnip cytochrome *f* which shows that the cluster of basic residues which probably interact with higher plant plastocyanin is too far from the haem for rapid electron transfer. Thus the initial, electrostatic complex is likely to undergo rearrangement to a different configuration prior to electron transfer.

Horse heart cytochrome *c* proved to be a good electron acceptor at low ionic strength. This protein is strongly

positively charged at its electron acceptor site, where a number of lysine residues form a positive ring around the solvent exposed haem edge [46]. The fact that the ionic strength does not show any effect on the reduction of cytochrome *c* below 60 mM salt suggests that if the reaction had a maximum, factors with a reverse effect would have a compensating effect on the interaction. These factors remain elusive at the moment. Control experiments (data not shown) with lettuce cytochrome *bf* complex and horse heart cytochrome *c* showed, as expected, that cytochrome *c* does not react with the higher plant *bf* complex at low ionic strength.

The *P. laminosum* plastocyanin sequence shows a number of significant differences when compared with the higher plant sequences. The small acidic patch (residues 59-61) is not present and the large acidic patch contains a lysine. It is therefore interesting to note that *P. laminosum* and higher plant plastocyanin show similar rates of reduction by the *P. laminosum* cytochrome *bf* complex at high ionic strength when electrostatic effects are negligible. Since the redox potentials of *P. laminosum* and spinach plastocyanin are similar [47], the driving forces for the two reactions are almost identical. The result is consistent with very similar structures for the close complexes active in electron transfer, and therefore suggests that the tunnelling pathway for reduction by cytochrome *f* is identical in the two plastocyanins.

Acknowledgements

The authors would like to thank M.E. Taylor for the preparation of *P. laminosum* cytochrome *bf* complex and plastoquinol-1. This work has been supported by grants from SERC and BBSRC. M.W. is supported by a BBSRC studentship.

References

[1] Hurt, E. and Hauska, G. (1981) Eur. J. Biochem. 117, 581–599.
[2] Kallas, T., Spiller, S. and Malkin, R. (1988) Proc. Natl. Acad. Sci. USA 85, 5794–5798.
[3] Widger, W.R. (1991) Photosynthesis Res. 30, 71–84.
[4] Mayes, S.R. and Barber, J. (1991) Plant Molec. Biol. 17, 289–293.
[5] Willey, D.L. and Gray, J.C. (1988) Photosynthesis Res. 17, 125–144.
[6] Mansfield, R.W. and Anderson, J.M. (1985) Biochim. Biophys. Acta 809, 435–444.
[7] Breyton, C., De Vitry, C. and Popot, J.-L. (1994) J. Biol. Chem. 269, 7597–7602.
[8] De Vitry, C. (1994) J. Biol. Chem. 269, 7603–7609.
[9] Stepphuhn, J., Rother, C., Hermans, J., Jansen, T., Salnikow, J., Hauska, G. and Herrmann, R.G. (1987) Mol. Gen. Genet. 210, 171–177.
[10] Martinez, S.E., Huang, D., Szczepaniak, A., Cramer, W.A. and Smith, J.L. (1994) Structure 2, 95–105.
[11] Martinez, S.E., Huang, D., Ponomarev, M., Cramer, W.A. and Smith, J.L. (1996) Protein Sci. 5, 1081–1092.
[12] Gray, J.C. (1992) Photosynthesis Res. 34, 359–374.
[13] Redinbo, M.R., Yeates, T.O. and Merchant, S. (1994) J. Bioenerg. Biomembr. 26, 49–66.
[14] Guss, J.M., Bartunik, H.D. and Freeman, H.C. (1992) Acta Cryst. B48, 790–811.
[15] Collyer, C.A., Guss, J.M., Sugimura, Y., Yoshizaki, F. and Freeman, H.C. (1990) J. Mol. Biol. 211, 617–632.
[16] Moore, J.M., Lepre, C.A., Gippert, G.P., Chazin, W.J., Case, D.A. and Wright, P.E. (1991) J. Mol. Biol. 221, 533–555.
[17] Bagby, S., Driscoll, P.C., Harvey, T.S. and Hill, H.A.O. (1994) Biochemistry 33, 6611–6622.
[18] Moore, J.M., Case, D.A., Chazin, W.J., Gippert, G.P., Havel, T.F., Powls, R. and Wright, P.E. (1988) Science 240, 314–317.
[19] Cookson, D.J., Hayes, M.T. and Wright, P.E. (1980) Nature 283, 682–683.
[20] Cookson, D.J., Hayes, M.T. and Wright, P.E (1988) Biochim. Biophys. Acta 933, 460–469.
[21] He, S., Modi, S., Bendall, D.S. and Gray, J.C. (1991) EMBO J. 10, 4011–4016.
[22] Modi, S., Nordling, M., Lundberg, L.G., Hansson, Ö. and Bendall, D.S. (1992) Biochim. Biophys. Acta 1102, 85–90.
[23] Led, L.J., Badsberg, U., Hammerstad, J.M., Jespersen, L. and Ulstrup, J. (1995) J. Inorg. Biochem. 59, 663.
[24] Cramer, W.A., Martinez, S.E., Furbacher, P.N., Huang, D. and Smith, J.L. (1994) Curr. Opin. Struct. Biol. 4, 536–544.
[25] Varley, J.P.A., Moehrle, J.J., Manasse, R.S., Bendall, D.S. and Howe, C.J. (1995) Plant Molec. Biol. 27, 179–190.
[26] Castenholz, R.W. (1970) Schweiz. Z. Hydrol. 32, 538–551.
[27] Gibson, T.J. (1984) Studies on the Epstein-Barr Virus genome, Ph.D. Thesis, Cambridge University, UK.
[28] Plesnicar, M. and Bendall, D.S. (1970) Biochim. Biophys. Acta 216, 192–199.
[29] Bendall, D.S., Bowes, J.M., Stewart, A.C. and Taylor, M.E. (1988) Methods Enzymol. 23, 327–344.
[30] Bendall, D.S., Davenport, H.E. and Hill, R. (1971) Methods Enzymol. 23, 327–344.
[31] Davenport, H.E. and Hill, R. (1952) Proc. Roy. Soc. Lond. B. 139, 327–345.
[32] Thomas, P.E., Ryan, D. and Levin, R. (1976) Anal. Biochem. 75, 168–176.
[33] Wallace, T.P., Stewart, A.C., Pappin, D. and Howe, C.J. (1989) Molec. Gen. Genetics 216, 334–339.
[34] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. Second edition, Cold Spring Harbor Laboratory, USA.
[35] Howe, C.J., Bowman, C.M., Dyer, T.A. and Gray, J.C. (1982) Molec. Gen. Genetics 186, 525–530.
[36] Devereux, J., Haeberli, P. and Smithies, O. (1984) Nucleic Acids Res. 12, 387–395.
[37] Dutton, P.L. (1978) Methods Enzymol. 54, 411–435.
[38] Wood, P. and Bendall, D.S. (1976) Eur. J. Biochem. 61, 337–344.
[39] Margoliash, E. and Frohwirt, N. (1959) Biochem. J. 71, 570–572.
[40] Stewart, A. and Bendall, D.S. (1980) Biochem. J. 188, 351–361.
[41] Tomioka, N. and Sugiura, M. (1983) Mol. Gen. Genet. 191, 46–50.
[42] Sykes, A.G. (1985) Chem. Soc. Rev. 14, 283–315.
[43] Gross, E.L. and Curtiss, A. (1991) Biochim. Biophys. Acta 1056, 166–172.
[44] Meyer, T.E., Zhao, Z.G., Cusanovich, M.A. and Tollin, G. (1993) Biochemistry 32, 4552–5449.
[45] Quin, L. and Kostic, N. (1993) Biochemistry 32, 6073–6080.
[46] Dickerson, R.E., Takano, T., Eisenberg, D., Kallai, O.B., Samson, L., Cooper, A. and Margoliash, E. (1971) J. Biol. Chem. 246, 1511–1535.
[47] Katoh, S., Shiratori, I. and Takamiya, A. (1962) J. Biochem. 51, 32.